FROST TOLERANCE AND WATER RELATIONS OF *BANKSIA* SPECIES IN TASMANIA PAST AND PRESENT

by

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Declaration

This thesis does not contain any material which has been submitted for the fulfilment of any other degree or diploma. To the best of my knowledge and belief, this thesis contains no material which has been published, written or provided by another person, except where due reference is given.

J. Blake

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ABSTRACT

Over the past several million years the diversity of *Banksia* species in Tasmania has declined. On the Tasmanian mainland today there are only two species of *Banksia* . These are the widespread and morphologically diverse *Banksia marginata* and*Banksia serrata* which has a very restricted to the State's northwest.

It is not clear when the decline in *Banksia* began in Tasmania, but Pleistocene sediments in western Tasmania demonstrate a relatively recent extinction for at least some *Banksia* species. The most recent recorded *Banksia* extinctions from Tasmania are *B. kingii* and *B. strahanensis.* These extinct fossil species are closely related to the extant mainland species *B. saxicola/B. canei* and *B. spinulosa/B. spinulosa* var. *cunninghamii* respectively.

Based on this information it is interesting that *B. marginata* managed to survive the climatic upheavals of the Quaternary in Tasmania while most other *Banksia* species were unable to.

This thesis investigates whether the extinction of *B. kingii* and *B. strahanensis* from Tasmania during the Pleistocene could be linked to a physiological incapacity to tolerate the climatic stresses of the Pleistocene glaciations, i.e. drought and cold.

A physiological examination of the drought and cold tolerance of the closest living relatives of the fossil species implies that the frost and drought tolerance of *B. kingii* and *B. strahanensis* were no less remarkable than that displayed by a sample of Tasmanian populations of the very successful *Banksia marginata.* It is therefore concluded that the Pleistocene extinction of *B. kingii* and *B. strahanensis* from Tasmania is unlikely to be due to a physiological weakness of drought and cold.

This study highlights the physiological mechanisms used *Banksia marginata, B. saxicola, B. canei* and *B. spinulosa* var. *cunninghamii* to survive climatic stress. All species were able to significantly increase their bulk elastic modulus in response to drought stress. *Banksia marginata* and *B. spinulosa* var. *cunninghamii* were also able increase their apoplastic water content in response to drought stress. The capacity for osmotic adjustment was not

characteristic of any of the species examined. It is interesting that all of the species examined underwent osmotic de-adjustment in response to drought stress.

Frost experiments indicate that all of the *Banksia* examined have reasonable frost tolerance. Although *B. spinulosa* var. *cunninghamii* was generally less frost tolerant than the other species, the results were usually significant. The frost results iimply that sensitivity of the fossil species to frost would have unlikely to cause their extinction from Tasmania during the Pleistocene glaciations. Indeed many of the *Banksia marginata* populations and *B. canei* and *B. saxicola* were able to significantly improve their frost tolerance when exposed to drought stress. The drought and frost experiments in this study successful highlight the similarities of drought and cold stress in their effect on plant physiology.

Banksia marginata was examined in more detail than the closest living relatives of the fossil species. This was done to help determine whether its current success in Tasmania is the result of its capacity for physiological plasticity and the genetic diversity of the species. The physiology results indicate that this species is very desiccation tolerant, thus giving it an excellent capacity to deal with drought and cold stress. The physiology results in general also indicated significant differences the drought and frost tolerance among populations of *B. marginata*. A small isozyme study was performed which suggested there could be a genetic basis to the physiological differences observed among two populations studied along an altitudinal cline.

An attempt was also made to determine whether the high degree of morphological variation evident in Tasmanian populations of *B. marginata* could be positively correlated with physiological variation, which it couldn't. Further work would need to done for this result to be accepted as conclusive.

It is likely that the success of *Banksia marginata* in Tasmania today is related to a combination of genetic diversity of the species, its physiological plasticity and its general hardy nature as evident from its extremely drought tolerant tissues.

The extinction of *B. kingii* and *B. strahanensis* from Tasmania during the Pleistocene may have resulted during the interglacial periods of the Pleistocene. Glacials are of a much longer duration than interglacials. Interglacials may have been very climatically stressful for the *Banksia*. Other hypotheses are considered in the thesis.

It is also likely that the genetic diversity of *B. kingii* and *B. strahanensis* was less than *B. marginata* during the Pleistocene. This theory is based on the current day morphological variation in the closest living relatives of the fossil species which is considerably less than displayed by *B. marginata*. In addition, the closest living relatives today have much narrower distributions than *B. marginata*, hence perhaps having less chance of surviving the climatic stress of the Pleistocene than the more widely dispersed and genetically diverse *B. marginata* which may have had greater genetic reserves for natural selection to act on.

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CHAPTER 1. INTRODUCTION

The History of *Banksia* in Tasmania

Plant macrofossils and palynological studies provide a window into the past from which information can be drawn about palaeofloras, palaeoclimatic conditions and plant species' extinction.

Over the past several million years the diversity of *Banksia* in Tasmania has undergone decline, e.g. at least seven species with *Banksia* type leaves were identified from a 35 million year old fossil deposit at Cethana in western Tasmania (Hill *et al.* 1992). Today, however, apart from the ubiquitous *Banksia marginata* Cav. there is only one other *Banksia* species present on the Tasmanian mainland, *B. serrata* L. f. which is restricted to a very small area in the north-west.

Banksia marginata is widely distributed throughout south-eastern Australia today, and is common in Tasmania, South Australia, New South Wales and Victoria (Fig. 1.1). Across its range, *B. marginata* displays great morphological variation, ranging from a small shrub less than 0.5 m in height, to a tree, taller than 10 m (Taylor and Hopper 1988). Its leaves vary enormously in size and shape (Fig 1.2). Even in the same general area there can be plants with different types of leaves and growth habits (Taylor and Hopper 1988), (Fig. 1.3).

Banksia marginata is the most common species of *Banksia* (Taylor and Hopper 1988). It is tolerant of a wide range of abiotic conditions and even on the fringe of its climatic range it appears to grow well. It occupies a wide range of soil types having a preference for sandy soils, although it is also found on clay loam, peaty loam, rocky soil, quartz, sandstone, limestone and granite (George 1981). It can occur in shrubland, woodland forest, swamps and coastal dunes within a rainfall range of 400-1000 mm per annum (George 1984). In Tasmania, *B. marginata* ranges from sea level to 1200 m altitude (Salkin 1979). It is common as a tree in western Tasmanian mixed forest and is found on the flood plains of the upper reaches of large rivers such as the Loddon, Franklin and Huon (Salkin 1979). It has also been recorded on the wet, acidic button grass plains of Phillips Lead, Huon Plains,

and Cox Bight (Salkin 1979), all coastal regions, Bruny Island, the Central Plateau and many other areas.

It is not clear when the decline in *Banksia* began in Tasmania but Pleistocene sediments in western Tasmania demonstrate a relatively recent extinction for at least some *Banksia* species (Jordan and Hill 1991). The most recent recorded *Banksia* extinctions from Tasmania are *B. kingii* and *B. strahanensis*, which have been described by Jordan and Hill (1991) from Pleistocene leaves and infructescences (Figs 1.4, 1.5 and 1.6). These species became extinct during the Pleistocene and are closely related to the extant *B. saxicola* A.S. George/*B. canei* J.H. Willis and *B. spinulosa* var. *cunninghamii* Sieber ex Reichenbach respectively, which are all currently found on the Australian mainland. In part, this study has sought to explain the extinction of *B. kingii* and *B. strahanensis* from Tasmania during the Pleistocene.

Furthermore, central to this study is the question of how *B. marginata* managed to survive, evolve and/or expand its range in Tasmania during the Quaternary while most other *Banksia* species became extinct from Tasmania.

There are **two** broad options available to account for this phenomenon:

(1). Banksia marginata represents a case of ecological release, i.e. where a single species freed from interspecific competition in an isolated land mass (Tasmania) has evolved to fill a broad range of available niches. However, it is unlikely that *B. marginata* represents a case of ecological release in Tasmania. This is because *B. marginata* also displays great morphological variation on the Australian mainland even though plant species diversity in general is far greater on the mainland than in Tasmania. Indeed, based on this, the scope for inter-specific competition on the Australian mainland should be far greater than in Tasmania.

(2). Banksia marginata is an inherently variable species (i.e. the evolution of this variability preceded its rise to prominence in Tasmania) and this variability allowed it to survive the Pleistocene and colonise niches made available through plant extinctions. Indeed, during the glacial events of the Pleistocene, the Tasmanian climate was much cooler and drier than at

present (Macphail 1978, Sigleo and Colhoun 1981, Bowden 1983), and a species had to be competitive under these conditions to survive.

Considering the unlikeliness that Option (1) is a suitable explanation for the success of *B. marginata* in Tasmania, Option (2) is the better working hypothesis. This hypothesis is framed on the assumption that *B. strahanensis* and *B. kingii* had more narrow distributions and less morphological and physiological variability (hence, perhaps less genetic diversity) in their populations during the Pleistocene than *B. marginata*. This assumption cannot be tested directly due to the limitations of the fossil record. However, all of the nearest living relatives of these fossil species have a more limited distribution (Figs 1.7, 1.8, 1.9) and morphological variation than current populations of *B. marginata*. Indeed, *Banksia marginata* has been described by George (1981) as the most widely distributed and most variable member of the genus. In other words, based on the range and morphological variation of the closest living relatives, it is assumed that the fossil *Banksia* would have had smaller distributions and/or at least less genetic variation than *B. marginata* during the Pleistocene. Consequently, with less genetic reserves to draw upon in times of physiological stress, the chance of each of the fossil species surviving in reproductively viable populations should have been comparatively less than for *B. marginata*.

It is also important to mention that there is no known fossil record of *B. marginata* for Tasmania and the Australian mainland. If this species was more widespread in the Pleistocene than the fossil species, it is interesting that there is no fossil record for it. Perhaps by chance *B. marginata* has not been fossilised anywhere in Australia.

Aims

The overall aim of this study has been to use plant physiology to determine:

(1). the possible cause(s) of the extinction of the fossil *Banksia* species, *B. kingii* and *B. strahanensis* from Tasmania during the late and early Pleistocene respectively (Jordan 1992) and,

(2). to account for the interglacial success of *B. marginata* in Tasmania today through:

a) an investigation of the physiological mechanisms used by this species to survive drought and frost stress;

b) determining whether the successful occupation of a wide range of habitats by this species is linked to genetic plasticity;

c) exposing any significant physiological and genetic differences among different populations of *B. marginata* in Tasmania and,

d) determining whether intrapopulation morphological variation in *B. marginata* is positively correlated with physiological variation, hence explaining its successful occupation of a wide range of habitats.

Evidence for the close relationship between the fossil *Banksia* species and extant *Banksia canei, B. saxicola* and *B. spinulosa* var. *cunninghamii.*

General

Banksia kingii sp. nov. fossils were found in sediments at Melaleuca Inlet, south-western Tasmania and *B. strahanensis* sp. nov. fossils were found in sediments at Regatta Point, western Tasmania (Jordan and Hill 1991).

Evidence for the close association between *1).* extant *B. canei/B. saxicola* and fossil *B. kingii* and, *2).* extant *B. spinulosa* var. *cunninghamii* and fossil *B. strahanensis* is based on the morphological assessments of leaf and cuticle specimens by Jordan and Hill (1991).

Cuticles and leaf sections were prepared from the *Banksia* macrofossils (Jordan and Hill 1991). The cuticles and leaf sections were photographed and were compared with a large range of extant material of tribe Banksieae including *Musgravea, Austromuellera,* all species of *Banksia* and most species of *Dryandra* (Jordan and Hill 1991). Jordan and Hill (1991) followed the taxonomy of George (1981) and Johnson and Briggs (1975).

Banksia kingii

Banksia kingii and extant *B. saxicola* and *B. canei* are illustrated in Figs 1.4 and 1.5. According to Jordan and Hill (1991) the robust symmetrical bilateral leaves with revolute margins and prominent midribs of *B. kingii* are typical of subtribe Banksiinae. Furthermore, within this subtribe entire leaves with prominent reticulate venation on the stomatiferous surface occur only in the *Banksia* series Salicinae (George 1981, Jordan and Hill 1991). Jordan and Hill (1991) maintain that *B. kingii* differs from all extant species in both microscopic and macroscopic features and requires specific status. It can however, be allied to *B. canei* and particularly *B. saxicola* on the basis of shared cuticular characters (Jordan and Hill 1991).

Banksia strahanensis

Banksia strahanensis and extant *B. spinulosa* are illustrated in Fig. 1.6. Jordan and Hill (1991) examined the fossil and extant species for the following leaf characteristics: leaf shape, margin shape, presence/absence of revolute margin, leaf width, leaf length, petiole length and apex shape. The non stomatiferous cuticle surfaces were examined for: papillae, cell size and cell shape. The stomatiferous cuticle surfaces were examined for: lamina hairs, veins and ribs. After examination of fossil leaf and cuticular specimens, Jordan and Hill (1991) concluded that the cuticular and leaf morphology of the fossil placed it readily in the subtribe Banksiinae, section *Oncostylis* of *Banksia*. *Banksia strahanensis* was subsequently compared with all the extant members of *Oncostylis*. Section *Oncostylis* is composed of three series: *Spicigerae*, *Abietinae* and *Dryandroideae*. Jordan and Hill (1991) concluded that the morphological characteristics of *B. strahanensis* are consistent with *Spicigerae* in all characters except that it does not show evidence of venation on the cuticle of the stomatiferous surface. According to Jordan and Hill (1991) *B. strahanensis* falls within the range of *B. spinulosa*. Jordan and Hill (1991) maintain that *B. strahanensis* is closely related to *B. spinulosa* but they are sufficiently different to warrant separate specific status.

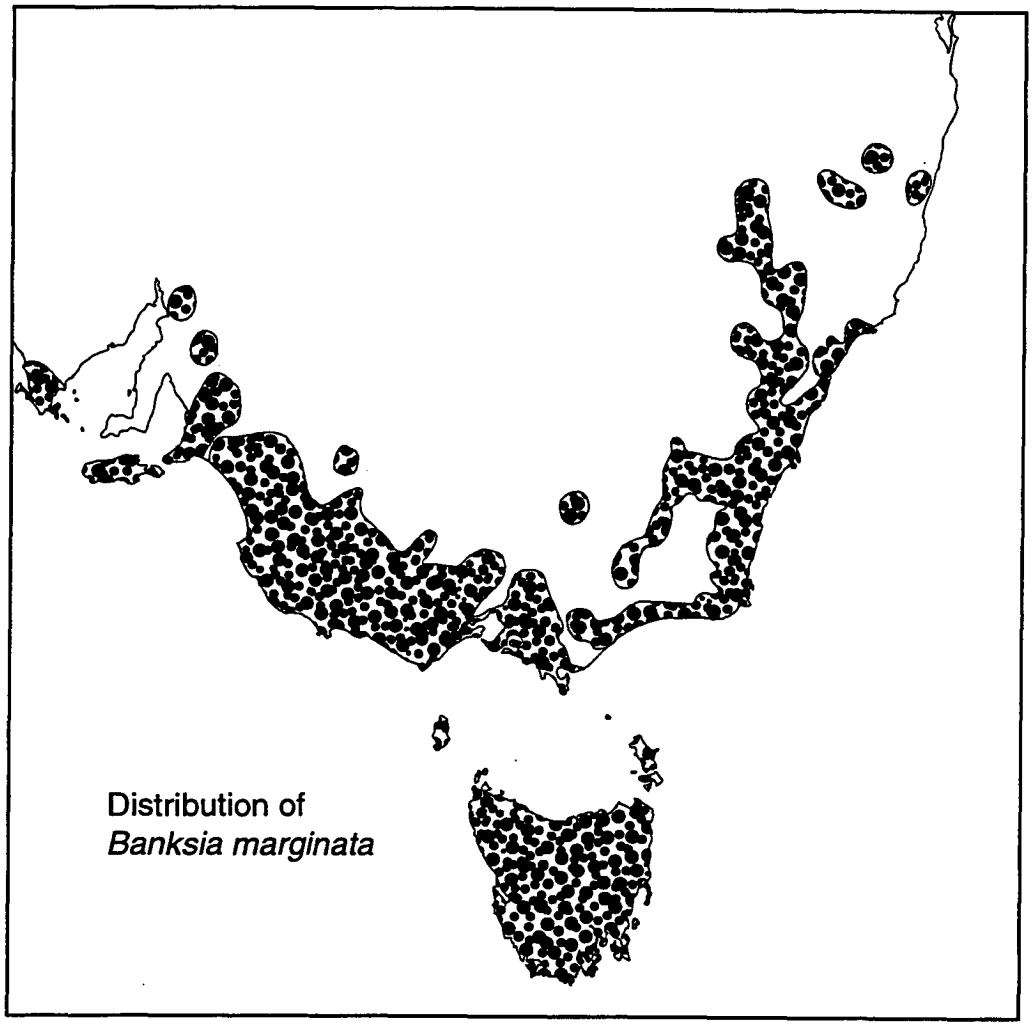

Figure 1.1. The distribution of *B. marginata* in Australia, adapted from Taylor and Hopper (1988).

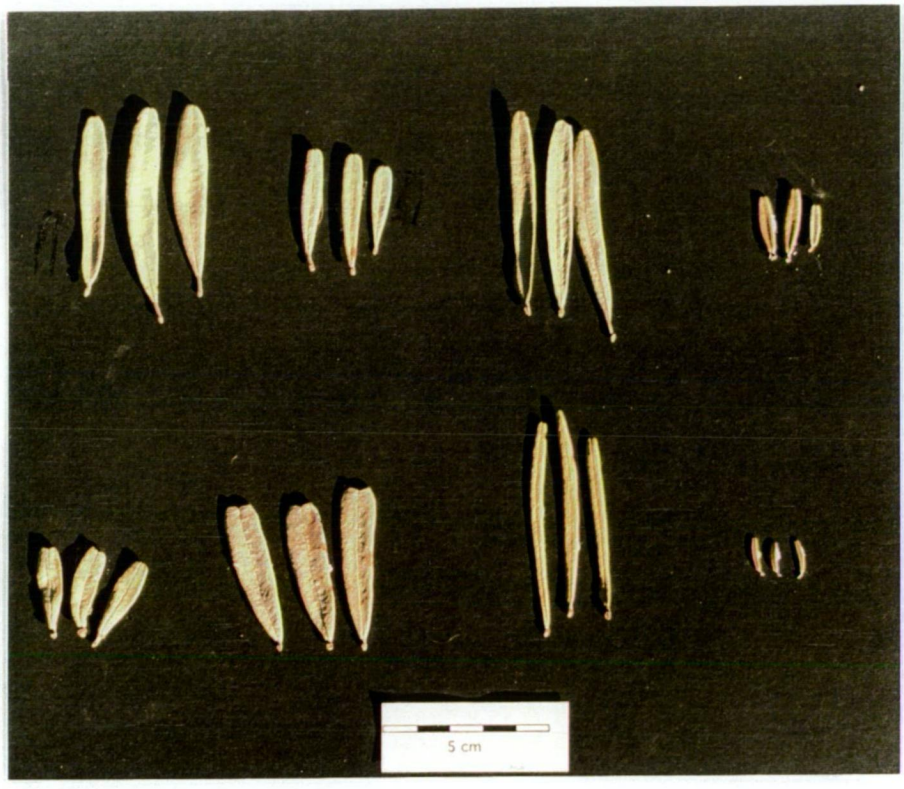

Figure 1.2. Examples of variation in the size and shape of *B. marginata* leaves from Tasmania.

Figure 1.3 (a). Examples of morphological variation in *B. marginata* from Freycinet National Park, Tasmania.

Figure 1.3 (b). Examples of morphological variation in *B. marginata* from Freycinet National Park, Tasmania.

Figure 1.3 (c). Examples of morphological variation in *B. marginata* from Freycinet National Park, Tasmania.

Figure 1.3 (d). Examples of morphological variation in *B. marginata* from Freycinet National Park, Tasmania.

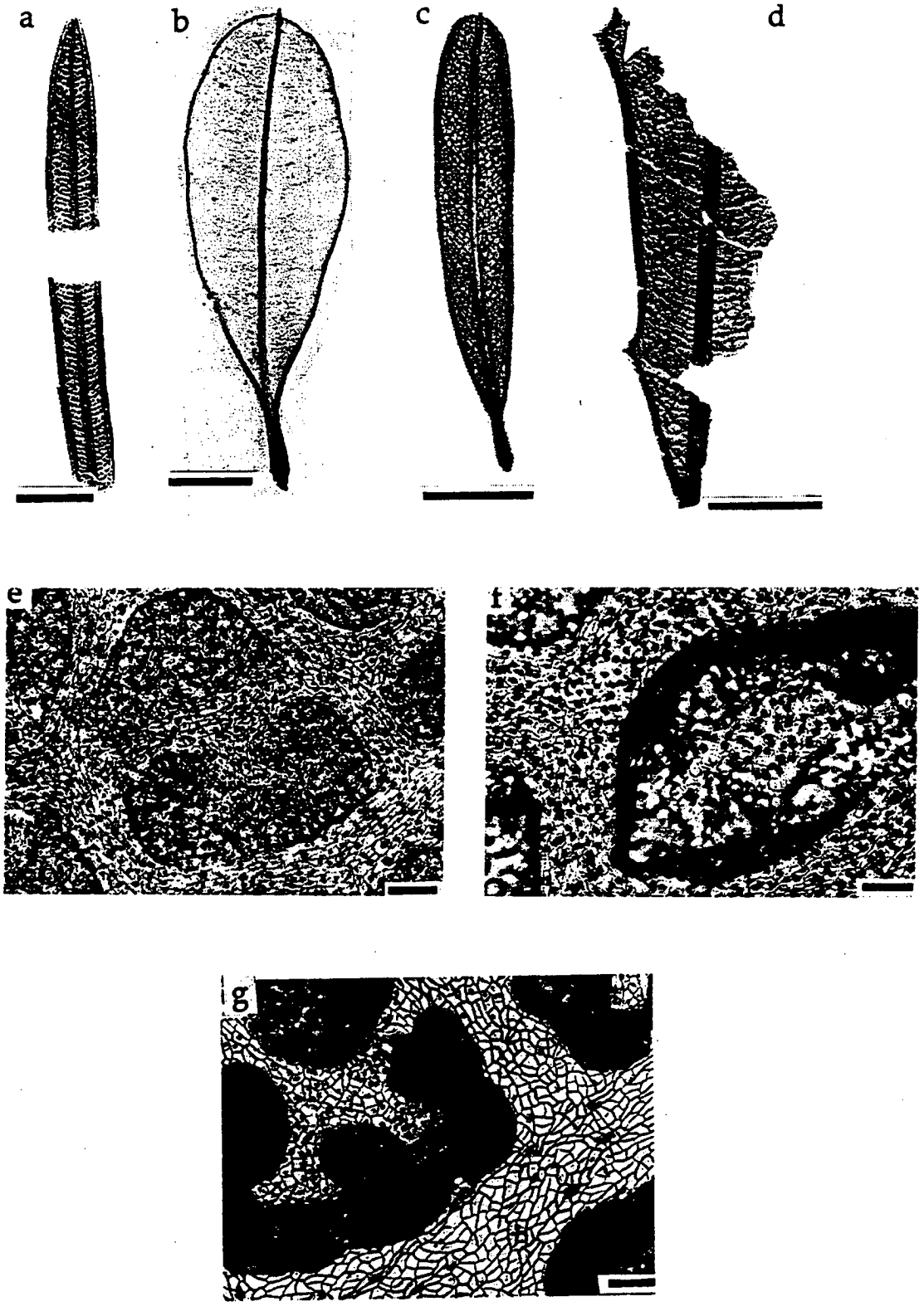

Figure 1.4. *Banksia* cuticle and leaves. a&e: Fossil *B. kingii* from Melaleuca Inlet. b&f: extant *B. saxicola*. c&g: extant *B. canei*. d: Fossil *B* cf. *kingii* from Regatta Point. Cuticles of the stomatiferous surface. Scale bars for a, b, c & d = 10 mm, for e, f & g =100 µm (from Jordan 1992).

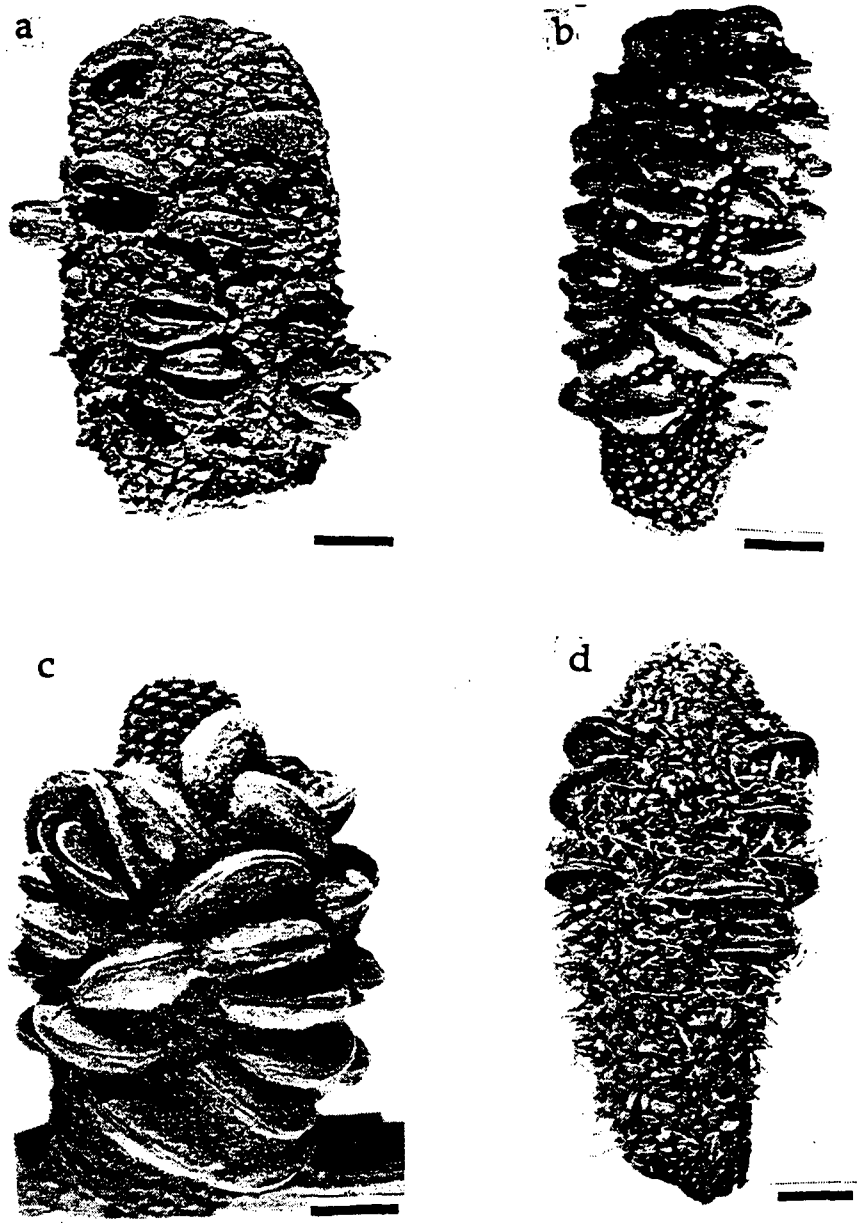

Figure 1.5.. *Banksia* infructescences. a: Probable *B. kingii* fossil from Melaleuca Inlet. b: Extant *B. saxicola*. c: Extant *B. canei* . d: Extant *B. marginata.* Scale bars for a, b, c & d = 10 mm (from Jordan 1992).

The mature *Banksia* infructescences are informally known as seed cones. The *Banksia* infructescence is a large woody structure with numerous follicles (George 1981). Once fully developed, the follicles may contain one, two or no viable seeds (George 1981).

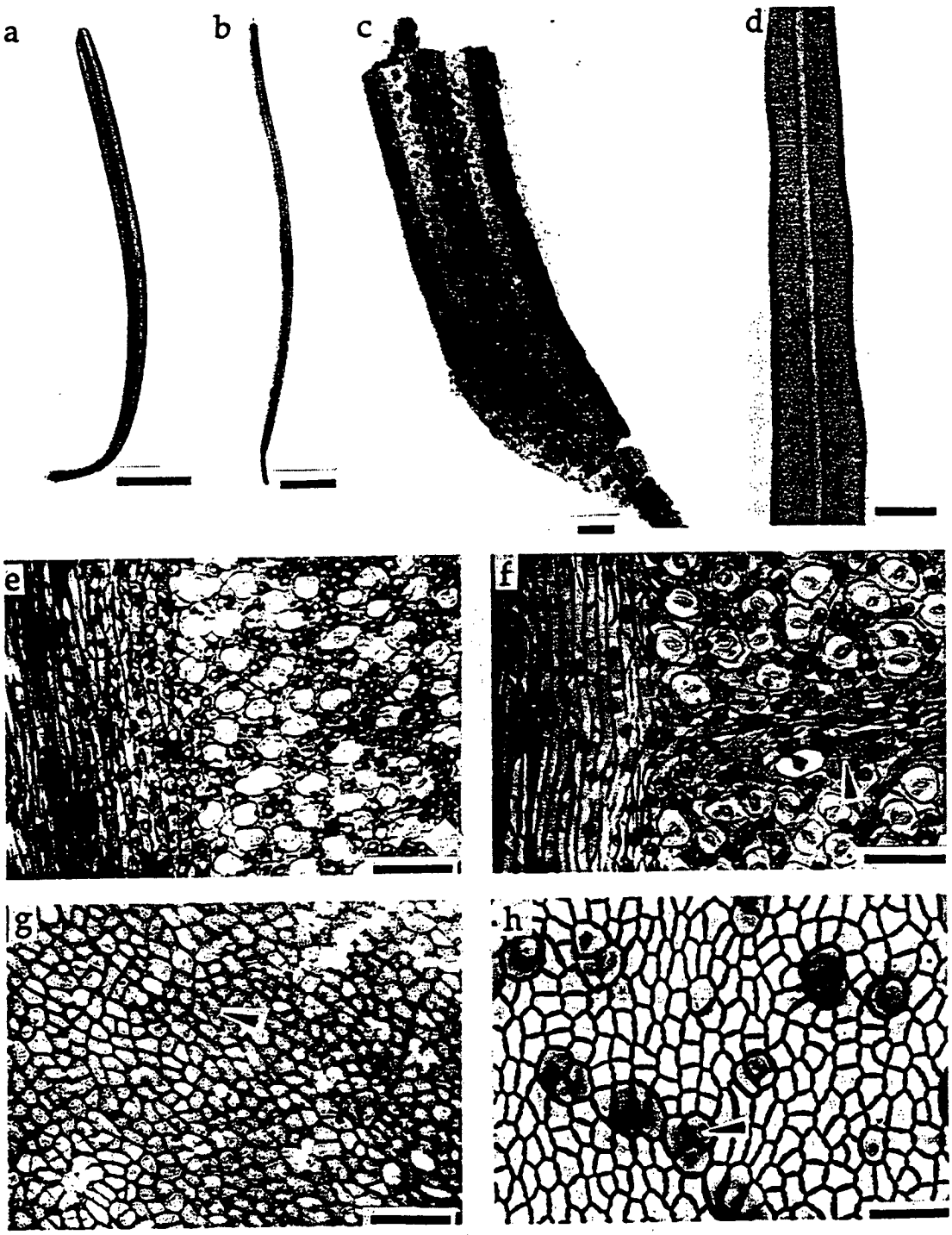

Figure 1.6. *Banksia* cuticle and leaves. a, c, e & g: Fossil *B. strahanensis* from Regatta Point. b, d, f & h: Extant *B. spinulosa*. a & b: Leaves. c&d: Leaf fragments. Scale bars for a & b = 5mm, for c & d = 2mm, for e & f = 100μm (from Jordan 1992).

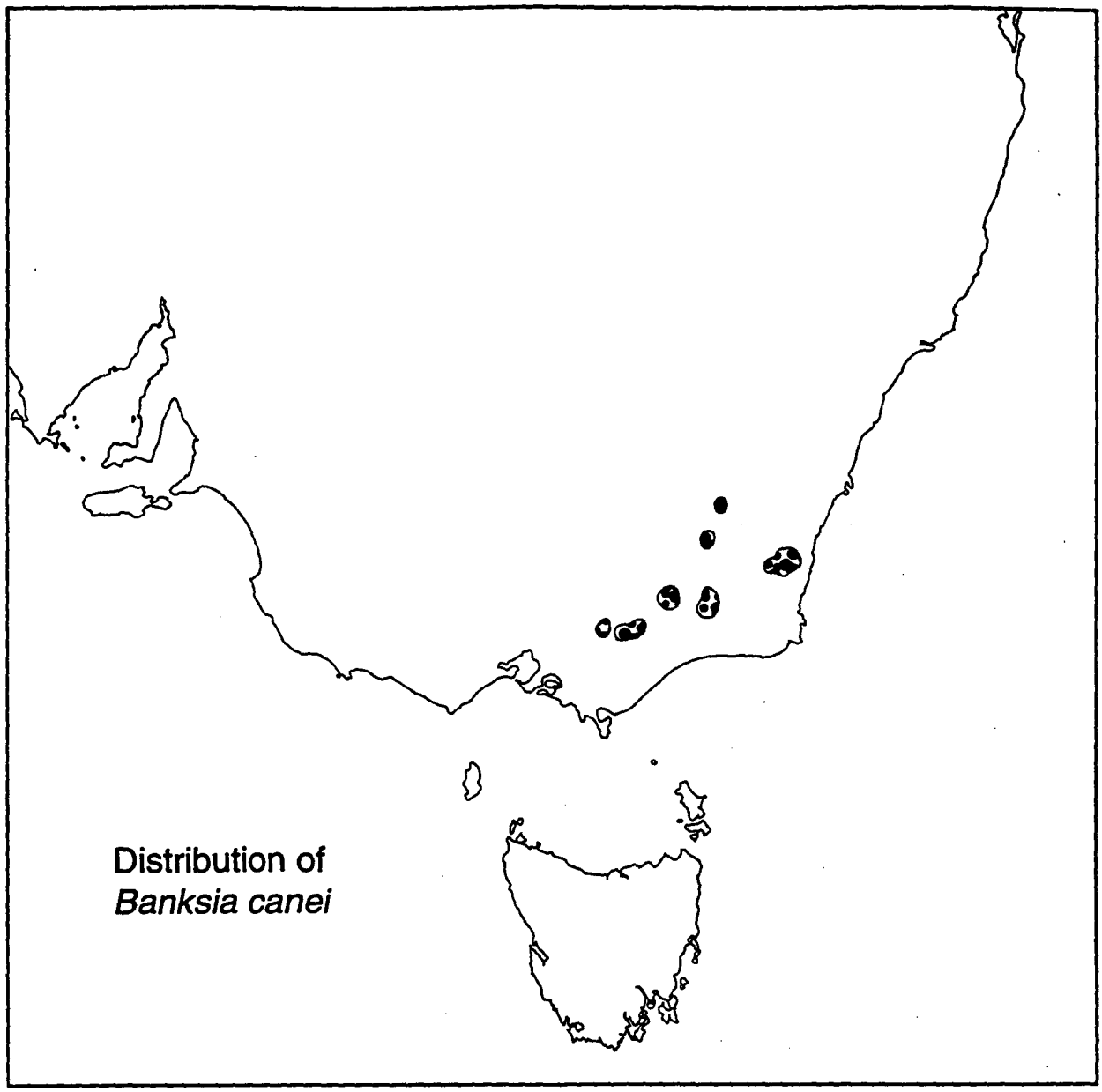

Figure 1.7. The distribution of *Banksia canei* in Australia, adapted from Taylor and Hopper (1988).

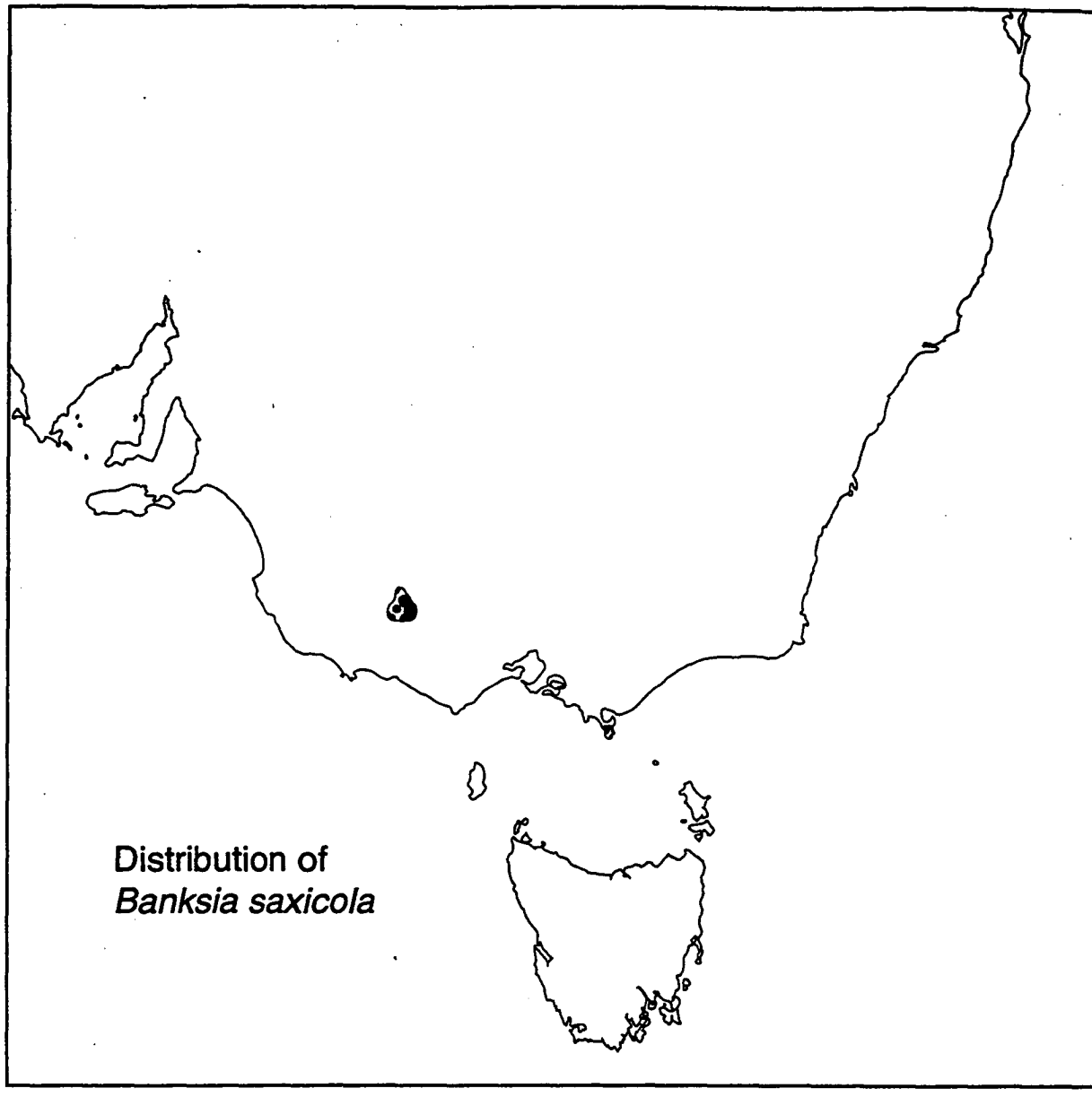

Figure 1.8. The distribution of *B. saxicola* in Australia, adapted from Taylor and Hopper (1988).

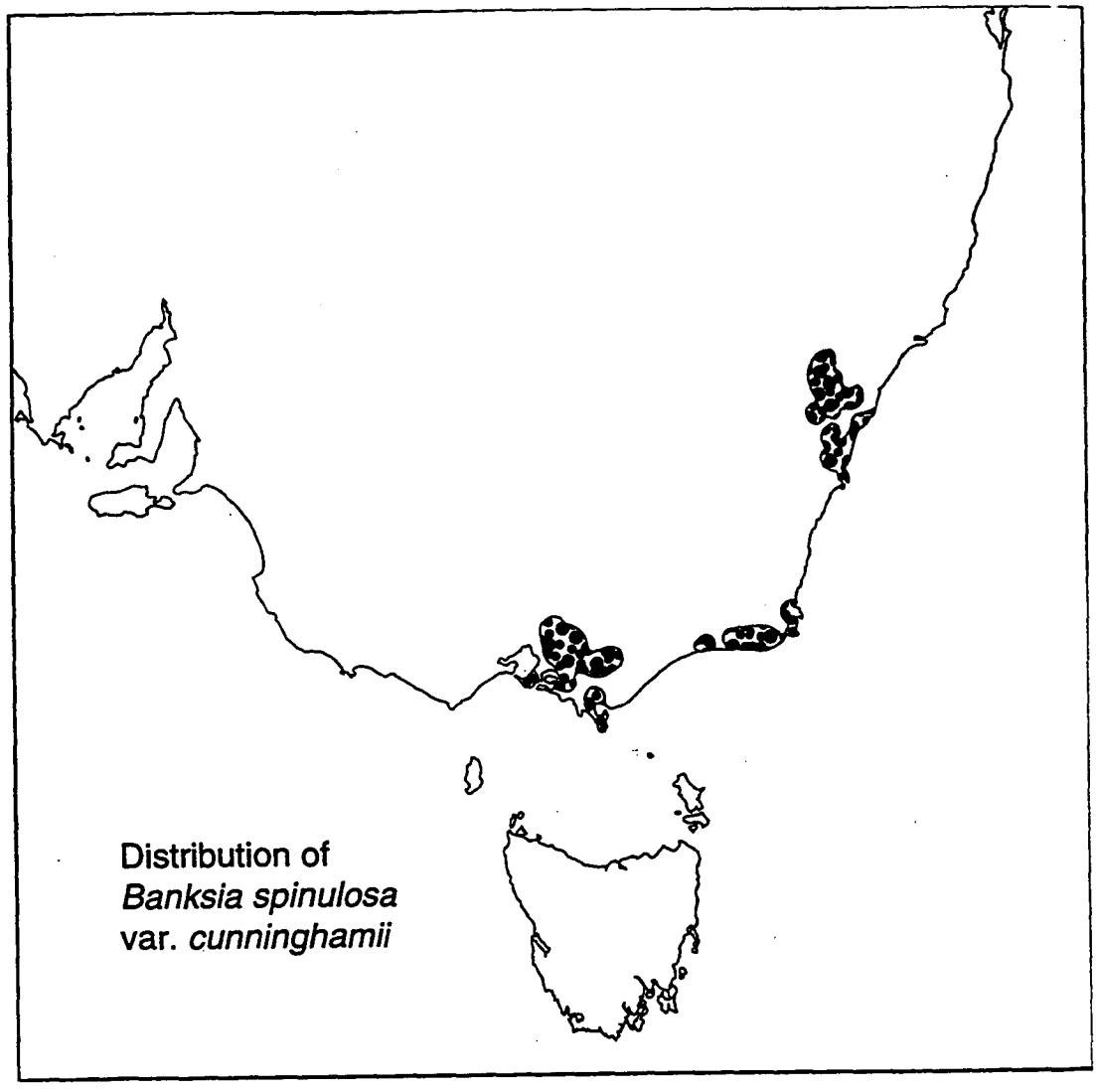

Figure 1.9. The distribution of *Banksia spinulosa* var. *cunninghamii* in Australia, adapted from Taylor and Hopper (1988).

CHAPTER 2. THE ROLE OF PLANT PHYSIOLOGY IN PALAEOBOTANY

Studies that predict fossil flora distribution and extinction during past climates often rely on the assumption that where there is similar morphology between a fossil specimen and an extant relative there should also be similarities in their physiology. Since it is not possible to compare the physiology of fossils with their extant relatives, this assumption relies on a certain degree of faith. However, there can be little dispute, that significant insight into extant, and therefore plant fossil ecology and physiology can be had from their morphology.

With regard to extant species there are many examples in the literature demonstrating the close link between physiology and morphology (e.g. Seddon 1974, Givnish 1987, Spicer 1989 and Groom *et al.* 1994). For example, a plant is termed xerophytic because both its morphology and physiology are geared toward reducing the amount of water lost through transpiration. In other words, one can be largely confident that any species exhibiting xeromorphy will be a water saver. The physiological control of stomatal aperture size when combined with characteristics typical of xeromorphy such as a dense layer of trichomes, protected stomates, wax, thick cuticle, sclerophylly, leaf size reduction and leaflessness all serve to reduce the rate of transpiration.

The close link between morphology and ecophysiology is further supported by Givnish (1987) who has detailed numerous trends in plant ecology and leaf morphology, for example: there is a trend for increased leaf size along gradients of increasing rainfall, humidity and soil fertility, reduction in leaf size with increasing irradiance and altitude, and increased leaf thickness with decreasing rainfall, humidity and soil fertility. Parkhurst (1978) has observed that amphistomatous leaves (stomata on both surfaces) are more frequent in sunny and/or dry sites than elsewhere.

Further evidence of the relationship between plant morphology and physiology is demonstrated through *Hakea trifurcata.* It has been revealed that this species has two leaf types, broad and terete, both occurring on the same plant but developing in different seasons (Groom *et al.* 1994). The two leaf forms have been found to have distinct physiological differences with

Note: *T50* refers to the temperature or desiccation level at which 50% tissue damage has occurred.

regard to their drought tolerance and photosynthetic capacities (Groom *et al.* 1994). It therefore stands to reason that if an extant species and its fossil relative share similar morphology they could also share similar physiology.

Givnish (1987) maintains that leaves provide an outstanding opportunity for comparative studies for numerous reasons, particularly because they are the primary site of photosynthesis and thus of fundamental functional importance to plants. This is very fortunate for palaeobotanists because leaves are commonly fossilised. Variation in leaf form, physiology and phenology has implications for carbon exchange and water loss. The balance between carbon exchange and water loss affects branching patterns, whole plant growth, and interactions with competitors, mutualists and predators (Givnish 1987).

Spicer (1989) maintains that potentially all life stages of land plants can be fossilised and that the physiological adaptation of plants to their habitats will be reflected to some extent in their morphology. It is therefore reasonable to take another step and make the assumption that the response of the closest living relative of a fossil species to a physiological stress, would be similar to the response expected from its fossil relative, where it is possible to make such a comparison. Prothero (1989) maintains that the basis for the extinction of many plant and animal species during the Late Eocene and Oligocene can be derived from understanding the ecological requirements of their extant relatives.

In this study I have considered the physiological response of the closest living relatives of certain extinct fossil *Banksia* species to climatic stress as a method to highlight any physiological weaknesses which may have led to their demise from Tasmania during the Pleistocene. To do this, the drought and cold stress tolerances of randomly selected Tasmanian populations of the very successful *B. marginata* were determined and used as a benchmark to compare the stress tolerances of the closest living relatives. Should the drought and cold tolerance of the closest living relatives have been significantly less than the *B. marginata* benchmark, this would have indicated a physiological weakness (for the purposes of this study). Physiological tolerances refer to the level of stress tolerance exhibited by a species, e.g. in terms of frost the *T50* values, and in terms of desiccation the *T50* values and in terms of the water relations the values for osmotic potential, water

potential, apoplastic water content and relative water content and water potential at turgor loss values (see later).

There is great scope for plant physiology to complement the work of palaeobotanists. Localised plant extinction due to historical factors e.g. climatic change, may be highlighted by using benchmark species to expose the physiological weaknesses of the closest living relatives of a fossil species. Only when the physiological tolerances and weaknesses of a species are identified can one appreciate the scope of its distribution under different climatic regimes. For example, some species may be better adapted to past conditions than existing ones, as is often the case with relict species. Blake and Jordan (1993) concluded from physiological studies that *Leptospermum grandiflorum*, which is currently restricted to small, disjunct patches at dry sites on the east coast of Tasmania, was likely to be more widespread during the dry Last Glacial than its current relict distribution suggests. Similarly, it has been suggested that the high frost resistance of *Nothofagus cunninghamii* is a retained feature reflecting its evolutionary and climatic history (Read and Hill 1989). For example, Hill and Read (1987) have suggested that this species may have evolved from an *N. moorei*-type ancestor during the late Tertiary, remaining in southern Australia during the cooling phase of this period where it was exposed to low temperatures. Sakai (1978) has also observed that some species of *Salix* have higher freezing tolerances than would be expected from their current climatic range. He suggests that this feature has been retained, having evolved under a cooler climate.

Other interesting physiological studies which have looked at the effect of Cenozoic climatic change on the distribution of plant species within Australasia have been done by Read and Hope (1989) and Read (1990) for *Nothofagus*. Today there are only three species of *Nothofagus* in Australia. However, during the Cenozoic, there were many more species. For example, during the Tertiary, there were *Nothofagus* species present in Australia whose closest living relatives are now restricted to New Zealand, New Guinea and South America (Hill 1990). Numerous authors have suggested that the low temperatures which developed during the late Tertiary resulted in the loss of the *N. moorei* type and the subgenus *Brassospora* species from south-eastern Australia (Specht 1981, Hill and Read 1987, Read and Hill 1989). However, Read (1990) has since concluded that

temperature change was unlikely to have stimulated the change in the distribution of *Nothofagus* within Australasia. She believes that the change in rainfall patterns in south-eastern Australia during the late Tertiary, from year round to the current winter dominated rainfall, may have resulted in the northward restriction of the larger leafed *N. moorei* and the current New Guinea species (Read and Hill 1989). Finally, she highlights the importance of further ecophysiological studies to palaeobotany by suggesting both the past and current distribution limits of the tropical and extra-tropical species of *Nothofagus* within Australasia may be explained through a study of the boundary layer resistance and water use efficiency of this genus.

Of course, there is more to species survival of climatically stressful events than simply their capacity to tolerate physiological stress, e.g. the survival of some species may be a function of chance. Furthermore, species exhibit varying amounts of intra-specific variation, both physiological and morphological. Those exhibiting large amounts of variation whether, genotypic or phenotypic, should have a better chance of surviving long-term climatic stress than species with less genetic diversity. The nature and importance of different types of variation are discussed later in other chapters.

In this thesis whole plant physiology is used to complement the work of the palaeobotanist, Jordan (1992). In particular, attention is given to *Banksia* extinctions, and the past and current distributions of certain *Banksia* species in Tasmania.

CHAPTER 3. DETAILS OF THE SPECIES STUDIED

The following species details have been adapted from Taylor and Hopper (1988) and George (1981 and 1984).

Banksia ***marginata*** Cavanilles (1800) (Silver Banksia): type collected by Luis Nee in March-April 1793, between Port Jackson and Paramatta, NSW.

Distribution: Widespread and often common in parts of South Australia, Victoria, New South Wales and Tasmania (Fig. 1.1).

Altitude: Ranges from sea level to 1200 m above sea level (asl).

Response to Fire: Some populations survive by lignotubers, or epicormic buds. Others are killed by fire and rely on regeneration from seed germination. Suckering has been recorded.

Morphology: Ranges from a shrub of 0.5 m to a tree greater than 10 m in height. Leaves vary enormously in size and shape and even in the same general area plants will often differ in form, leaf and cone shape and size. It is not known to what extent these differences are genetic or plastic. The adaxial surfaces of the leaves range from dark to medium dark green. The abaxial surfaces are always white with many trichomes. At maturity the old perianths and styles remain on the seed cones.

Flowering Period: Mainly February-July.

Rainfall: 400-1000 mm/annum.

Frost Tolerance: Often frost tolerant.

Growth: Relatively fast growing, subalpine forms require seed stratification (i.e. seed should be stored for at least 60 days at 5°C prior to germination).

General comments on *B. marginata*

Banksia marginata has long proven to be a problematic species with respect to its taxonomy, and has a history of being subdivided into different species. For example, Robert Brown (1810) divided *B. marginata* into six different species: *B. marginata*, *B. microstachya*, *B. depressa*, *B. patula*, *B. australis* and *B. insularis*. George (1981) indicates that *B. marginata* has also been subdivided into *B. ferrea* Sprengel (1825), *B. gunni* Meissner (1856), *B. integrifolia* Meissner (1856), *B. marcescens* Bonpl. (1816), *B. marginata* R.Br. (sic) var. *cavanillesii* Endl (1856), and *B. marginata* R.Br. (sic) var. *humilis* Meissner (1856). The current taxonomic status of *Banksia marginata* is based on the work of George (1981) who states "many variants have been named but I accept none of them even at a varietal level". He also maintains that "the species warrants a detailed study including field work, but this would require a major task in view of the large area it covers."

Banksia marginata is animal pollinated and hybridises readily with other species in the genus, with introgressive hybridisation occurring at times, i.e. where genes infiltrate from one genotype to another (Salkin *circa* 1983). The following mainland species have been observed to hybridise with *B. marginata*: *B. paludosa*, *B. saxicola*, *B. integrifolia* var. *integrifolia* (Taylor and Hopper 1988) and *B. conferta* var. *penicillata* (George 1984). Thus, at least some of the morphological variation in mainland *B. marginata* may be attributed to hybridisation. However, in Tasmania there are no *Banksia* species other than the very localised *B. serrata* for *B. marginata* to hybridise with. There are no published accounts of *B. marginata/B. serrata* hybrids. Thus, the morphological variation evident in Tasmanian *B. marginata* should not be a case of recent hybridisation events. The variation may however, have an historical origin, resulting from the hybridisation of *B. marginata* with the *Banksia* species present in the State prior to their extinction.

Banksia canei J.H. Willis (1967) (Mountain Banksia), close living relative of the fossil species *B. kingii*. Type collection by Jim Willis near Wulgulmerang, eastern Victoria, November 27, 1962 .

Distribution: Eastern Victoria, south-eastern NSW, in subalpine areas between the Snowy Range and Gippsland and Tuross River, usually between 750-1500 m asl, (Fig. 1.7).

Habitat: Occurs on rocky, granitic or sandstone soil, on slopes and in gullies in low open woodland and open heath.

Response to Fire: Unknown, but probably fire sensitive.

Morphology: A much branched, erect or spreading shrub up to 4 m in height. Adaxial surface of the leaves dark green with white abaxial surfaces. The mature flowers fall off the seed cones leaving them bare.

Flowering: December-May, peak February-April.

Rainfall: 800-1200 mm/annum.

Frost: Frost tolerant.

Growth: Slow growing shrub, five to seven years from seed to flower.

General comments on *B. canei*

When Ferdinand Mueller first collected *B. canei* in 1853 he called it *B. marginata*, however in 1963 Jim Willis re-named it *B. canei* (Salkin *circa* 1980).

Salkin and Hallam (1978) have described four distinct geographically isolated forms of *B. canei*. The topodemes relate to the following four geographic regions: The Kybean range topodeme, Snowy Mountains topodeme, Coastal topodeme and Grampians topodeme (Salkin and Hallam 1978).

At the time of the *B. canei* seed collection, I was unaware of the significance of the four topodemes. It is unfortunate that not all of the different forms of *B. canei* were tested.

Figure 3.1. *Banksia canei*, taken at Omeo in Victoria (1993).

Banksia saxicola A.S. George (1981), close living relative of *B. kingii*: Type collected from Mount William in the Grampians, Victoria, by Alex George in 1977.

Distribution: Has a restricted distribution occurring in two disjunct populations, the Grampians (western Victoria) and Wilsons Promontory (Fig. 1.8).

Habitat: In the Grampians it occurs as a shrub up to 5 m in height, on slopes and mountains, on sandy, loamy, and rocky soils. At Wilsons Promontory it typically ranges between 3-8 m in height amongst sand and granite rock in eucalypt forest and woodland.

Altitude: Most of the population at Wilsons Promontory occurs near sea level to 300 m asl, inland from Sealers Cove. In the Grampians it generally grows above 550 m and has been recorded as high as 1168 m on the summit of Mount William.

Response to Fire: Generally killed by fire, but regenerates from seed.

Morphology: As mentioned previously, it may grow as a tree (e.g. at Wilsons Promontory) and a shrub no more than 2 m tall on the exposed slopes of the Grampians. Its leaves occur in whorls. Leaf adaxial surfaces are dark green and the abaxial surfaces are white and covered in many trichomes.

A presumed hybrid of *B. saxicola* and *B. marginata* has been recorded near the summit of Mount William in the Grampians. This plant has leaves and cones intermediate between *B. saxicola* and *B. marginata*.

Flowering: January-March.

Rainfall: 700-800 mm/annum.

Frost: Frost tolerant.

Growth: Probably takes up to six years to flower. Seed needs stratification for at least 60 days at 5°C prior to germination.

General comments on *B. saxicola*

The two disjunct populations of Wilsons Promontory and the Grampians may be taxonomically distinct.

Figure 3.2. *Banksia saxicola* taken at Mount William, Grampians Victoria.

Banksia spinulosa var. ***cunninghamii*** Sieber ex Reichenbach, close living relative of the fossil species *B. strahanensis*: Type collection from Mount York, Blue Mountains NSW, by Franz Sieber in 1923.

Distribution: Found in south-eastern Queensland, in NSW along the Great Dividing Range south to the Shoalhaven River, and in Victoria from the Dandenongs and Wilsons Promontory east to the NSW border (Fig. 1.9).

Habitat: Occurs on sandy soils, sometimes overlying rock, and also heavier loams and clays. It is found as an understorey plant on hillsides in *Eucalyptus* woodland and forest.

Altitude: Occurs from near sea level-1000 m asl.

Response to Fire: Non lignotuberous, killed by fire, regenerating from seed.

Morphology: A shrub or tree up to 4 m in height, sometimes larger. Can be either bushy or sparse in foliage. The leaf adaxial surfaces are usually deep green and the abaxial surfaces white with many trichomes. The old flowers fall from the seed cones leaving the cones bare.

Flowering: April-August.

Rainfall: 750-1200 m.

Frost: Tolerates frost to -8°C.

Growth: Fairly fast growth, usually taking between five to six years from seed to produce flowers.

General comments on *B. spinulosa var. cunninghamii*

Banksia spinulosa var. *cunninghamii* belongs to the *Oncostylis* complex which is totally extinct from Tasmania (Jordan 1992). *Banksia spinulosa* var. *cunninghamii* is one of four varieties of *B. spinulosa* which have been

recognised *(1).* var. *cunninghamii, (2).* var. *spinulosa, (3).* var. *collina, (4).* var. *neoangelica.* The varietal classification of *B. spinulosa* has been under recent review by Peter Weston of the Botanical Gardens of Sydney, New South Wales.

Figure 3.2. *Banksia spinulosa* var. *cunninghamii,* Sealers Cove Walking Track, Wilsons Promontory Victoria, taken in 1993.

General comments on the species used in this study.

It is interesting that two of the closest living relatives of the Pleistocene fossil *Banksia* species under investigation are either directly or indirectly related to *B. marginata*. For example, *B. marginata* is closely related to *B. canei*. *Banksia canei* is similar to *B. marginata* in showing some variation in leaf morphology, though this is far less variable in this regard than *B. marginata*. Salkin and Hallam (1978) have suggested that *B. canei* may have once shared the same gene pool as *B. marginata*, with gene flow having since been blocked by its isolation at high altitudes. Salkin and Hallam (1978) have found four topodemes of *B. canei*.

Banksia saxicola is related to *B. canei* and *B. integrifolia*.

Banksia spinulosa var. cunninghamii is related to *B. ericifolia* and *B. occidentalis* (George 1984) and is the most morphologically variable of the fossil relatives examined.

CHAPTER 4. WATER RELATIONS AND FROST METHODOLOGIES

Introduction

Information on regional climate is based on years of accumulated weather observations. However, the data base for known measurements of regional weather for Australia is limited to the last 200 years. Consequently, information on past Australian climates has to be derived with less direct means such as from the fossil flora, land formations, stratigraphy, radiocarbon dating, percentages of indicator micro-organisms in deep sea cores, and oxygen-isotope ratios to mention a few.

In short, plants depend on climate and soil (Strahler and Strahler 1983), with different species of plant having different water and temperature requirements (Mielke 1989). Indeed, climate forms the basis for defining the physical regions of the earth (Strahler and Strahler 1983) and this is reflected by the order of plant communities across the globe which are adapted to the climatic zones they occupy (Mielke 1989). Furthermore, the earth's biota has been grouped into several ecological associations called biomes which more or less correspond to the climatic zones of the earth e.g. tundra, boreal forest, prairie, steppe, desert, chapparal, broad leaf-mid latitude forest, mixed broad leaf coniferous forest and mountain ranges (Mielke 1989). Plants which occupy each of these biomes have a characteristic range of temperature and precipitation that they can tolerate (Mielke 1989).

Regional weather is a function of latitude and tropospheric large scale air motions and air mass interactions (Strahler and Strahler 1983). The small scale dynamic nature of weather is most clearly displayed at higher latitudes where obvious seasonal, daily, hourly, even minutely fluctuations in weather occur. These small scale, regional fluctuations in weather all affect plant survival and reproduction.

Regional weather is also strongly affected by the global climate. For example, temperature and precipitation are largely dependent on whether the global climate is in a glacial or an interglacial. Cyclic glacial and interglacial

events have affected the survival of plants since the Permian at least. As mentioned previously, the two dominant forms of climatic stress which affect plants during a glacial event are drought and cold. Species that can adapt to or tolerate changing climates survive, those which cannot become extinct, migrate or retract to refugia.

Directional shifts in both mean annual temperature and precipitation as well as an increased probability that extreme climatic events will occur, can profoundly alter ecosystem type, community structure and ecosystem processes (Pastor and Post 1986). Ecosystem response to changes in temperature and water availability can result in shifts in stand dominance, as well as changes in the physiological resource-use efficiencies of many species (Lajtha and Gertz 1993).

Since the extinction of *B. kingii* and *B. strahanensis* are thought to have occurred during the Pleistocene (Jordan 1992), a time in which at least four glaciations occurred, the frost and water relations of the closest living relatives of the fossils (*B. spinulosa* var. *cunninghamii*, *B. canei*, *B. saxicola*) have been examined as part of this study. These results were compared to the frost and water relations of eight Tasmanian populations of the apparently successful *B. marginata*. From these results an attempt is made to extrapolate what the field survival of each of these species may have been like during the climatically stressful glacial events of the Pleistocene.

General Methods and Materials

Seed collection

Seed was collected from ten randomly selected parent plants for the following species and sites from Victoria in June 1992 (Table 4.1):

Table 4.1. List of Victorian *Banksia* species for which seed was collected.

Species	Site	Altitude
Banksia canei	Omeo	~750 m
Banksia saxicola	The Grampians, Mount William	~1000 m
Banksia saxicola	Wilsons Promontory	~150 m
Banksia spinulosa var. *cunninghamii*	Wilsons Promontory	~200 m

Seed was collected from five to ten randomly selected parent plants of *B. marginata* from the following Tasmanian sites in June 1992 (Table 4.2, Fig. 4.1):

Table 4.2. List of sites from where seed was collected from *B. marginata* in Tasmania.

Banksia marginata	Bathurst Harbour	~ 20 m asl
Banksia marginata	Birches Inlet	~ 20 m asl
Banksia marginata	Snug Plains	~600 m asl
Banksia marginata	The Gardens	~10 m asl
Banksia marginata	Cape Hauy	~80 m asl
Banksia marginata	Cape Tourville	~100 m asl
Banksia marginata	Mount Wellington	~1040 m asl
Banksia marginata	The Blow Hole	~100 m asl

Random sampling technique

The following technique was used to randomly select plants to collect seed from *Banksia canei*, *B. saxicola* from Mount William and Wilsons Promontory, *B. spinulosa* var. *cunninghamii*, *B. marginata* - Snug Plains, Cape Hauy, Cape Tourville, Mount Wellington and the Blow Hole.

At each site for each species, 30 *Banksia* trees were randomly allocated a number from 1-30. Ten numbers were then randomly generated from a total of 30 possible numbers. The trees were then randomly sampled for seed according to their numbers.

I did not collect the seed of *B. marginata* from Bathurst Harbour, Birches Inlet and The Gardens. From these sites between 5-10 trees were randomly sampled for seed (K. Williams pers. comm.).

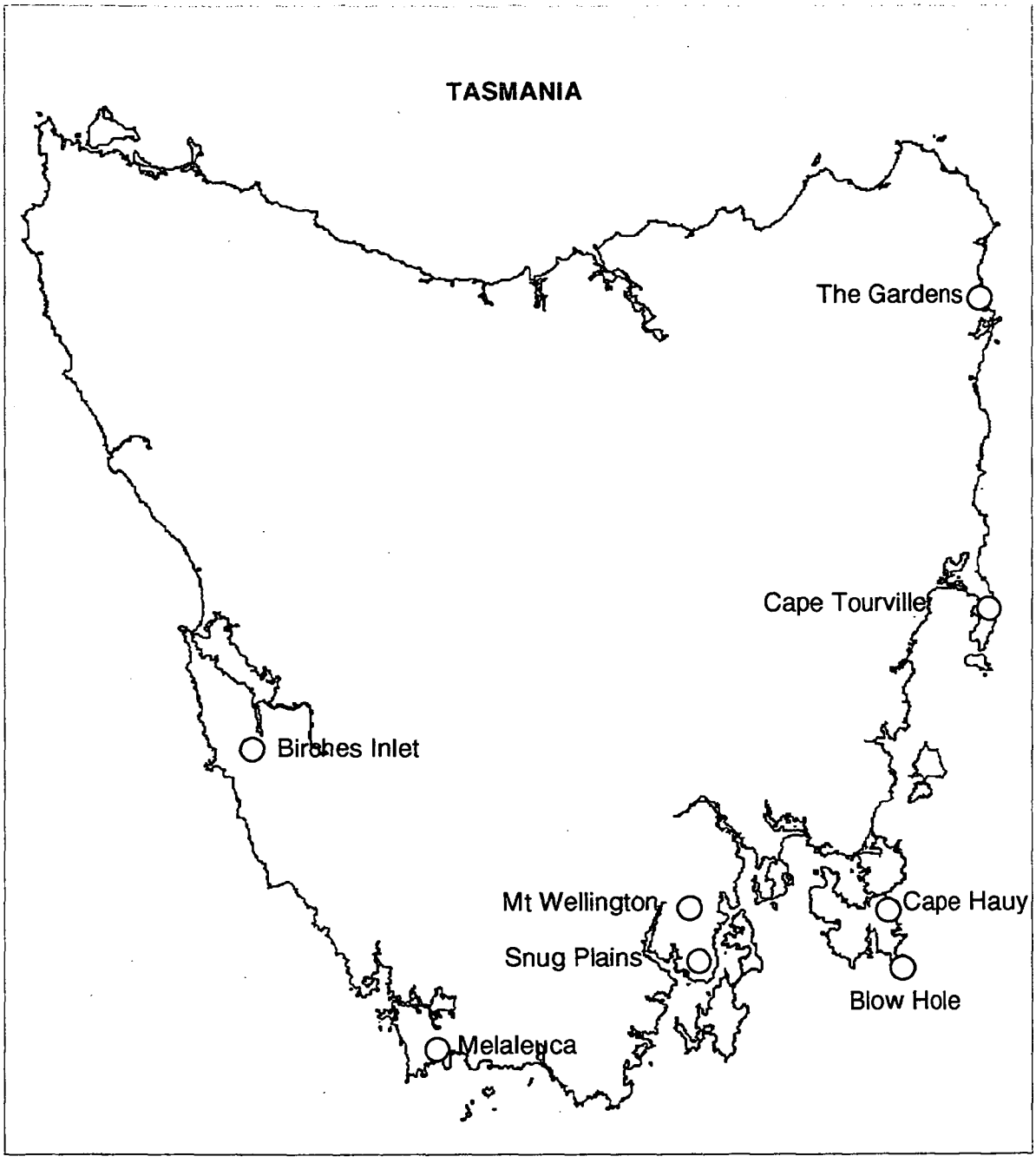

Figure 4.1. Locations of sites where *B. marginata* seed was collected from in Tasmania.

Germination

Seed was sown on a bed of steam sterilised soil consisting of 4:3 parts sand and peat respectively. After sowing, seed trays were either soaked with water and placed in the glasshouse for germination or were soaked and placed in a cool room for stratification. For example, sub-alpine *B. marginata* seed collected from Mount Wellington and Snug Plains and *B. saxicola* from Mount William and *B. canei* from the Omeo Highway were stratified in a dark room at constant temperature of 5°C for 3.5 months as suggested by Mullet (1982).

Once the seedlings had developed between four to six leaves they were potted up into 5 cm diameter, 6.5 cm high and later 7 cm diameter, 10.5 cm high pots. The soil for raising the seedlings was steam sterilised and comprised of sandy loam, peat moss, and river sand in the ratio 4:3:2 respectively. The nutrient status of the potting mix was enriched with 60 ml of dolomite and 120 ml of blood and bone in 4 l lots. Seedlings were grown in the glasshouse and watered daily over spring, summer and autumn, over the winter they were watered every second day.

Why Study the Physiology of Seedlings?

Seedlings are commonly used for laboratory based physiology experiments (e.g. Bachelard 1986, Read and Hill 1988, Wang *et al.* 1988, Vance and Zaerr 1991). They are practical where there are time constraints (as in this study), although cuttings from adult plants can be struck successfully for certain species. Cuttings were not a practical option for this study, mainly with regard to quarantine matters (i.e. Victorian collected specimens) and the time associated with transporting them to Tasmania. The study of seedlings (and cuttings) from properly designed studies where the original parent plants can be referred back to, can help determine whether the phenotypic traits of the progeny are genetically fixed or plastic.

By examining the physiology of seedlings, it is possible to collect information on species replacement in a field situation. Further to this, by exposing a species to various types of physiological stress it is possible to determine their physiological hardiness and therefore extrapolate the likelihood of their

survival during seedling establishment phase while under climatic stress (Blake and Jordan 1993). Indeed, it has been widely accepted that a tree species whose seedlings can survive and grow steadily in a field situation has a good chance of becoming canopy species in time (Woods 1979).

Some authors do not think that the extrapolation of the behaviour of laboratory tested, glasshouse seedlings to the field is legitimate because the differences between the abiotic/biotic conditions of the field and lab are too great. To improve confidence in laboratory experiments, some authors experiment on field plants to compare the results with glasshouse plants, e.g. (Robichaux 1984, Collier and Boyer 1989, Cook and Ladiges 1991). This is an excellent, though not always practical way of checking results. For this thesis, field experiments were not practical because of the absence of the mainland subject species in Tasmania. Where it is possible to carry out both field and laboratory experiments it is important not to generalise the results. For example, the physiology results of seedlings may not be directly comparable to those of adult plants. Additionally, consideration must be given to the field conditions (e.g. soil water potential, soil type and structure, air temperature, sun or shade habitat etc.) that the field plants had been exposed to prior to their testing to determine how comparable the results are.

Note: Different species can be subject too the same experimental conditions and the responses compared, even if lab conditions are not the same as the field. The differences are still valid information about the plant's performance under defined conditions.

Polyethylene Glycol 3350 as an Osmoticum

For the drought experiments described in Chapter 5 and some of the frost experiments in Chapter 6, plants were drought stressed within a hydroponic system using polyethylene glycol (PEG) from the Sigma Chemical Company, average molecular weight of 3350. Droughting the plants hydroponically with PEG ensures that all plants regardless of size will be drought stressed to the same extent. This has the effect of removing any experimental error associated with differential droughting that occurs when plants of different sizes i.e. with different root/shoot ratios are droughted in a confined space of soil.

PEG has been observed to lack many of the undesirable properties associated with other osmotica. Consequently, it has been used by numerous workers in water relations studies (Zwaizek and Blake 1989, Perez-Alfocea *et al.* 1993, Erdei and Taleisnik 1993, and Tan and Blake 1993). For example, PEG is not easily broken down by living organisms and has been shown to be non-toxic in most cases (Mexal *et al.* 1975). Furthermore, PEG molecules are not readily absorbed by intact plants (Janes 1968, Lawlor 1970 as cited in Mexal *et al.* 1975) and molecules with a molecular weight greater than 3000 are apparently not absorbed at all (Tarkow *et al.* 1966 as cited in Mexal *et al.* 1975). Mexal *et al.* (1975) did indicate that plants growing within nutrient solutions containing PEG may suffer from oxygen deficiencies. To prevent this, they recommend either limiting the PEG concentration or supplying considerable aeration to the system. They have also found that oxygen dissolution in PEG 4000-6000 solutions is inversely related to the molecular weight of the PEG concentration.

For the hydroponic drought experiments, PEG 3350 was used for its low molecular weight, the molecular weight however, was not so low that its molecules could be absorbed by the plants. Mexal *et al.* (1975) have recommended that the choice of PEG for an osmoticum should be based on both the molecular weight of the PEG and its properties in solution.

The Hydroponic System

Plant roots were shaken free of soil and washed in water. Whole plants were then placed in 7 cm diameter pots and filled with coarse gravel to cover the root system. Nine extra holes were drilled into the body of the pots to facilitate water flow through to the plant roots. The pots were randomly placed in a plastic tub 50x33x13cm with 9 litres of half strength Hoagland's nutrient solution and the plants were grown hydroponically prior to experimentation. The nutrient solution was circulated through the system via a pump and additional aeration was provided through two aquarium pumps with a total of eight, 8 mm diameter tubes blowing air into the system.

pH was monitored every second day. The pH ranged between six and seven. When the pH became too alkaline, drops of 10% H_2S0_4 were added. See Fig. 4.1 for the hydroponic system.

It is likely that the coarse gravel would have impeded fluid circulation around the roots, therefore reducing the quantity of solution reaching the roots and perhaps increasing the time that a certain amount of solution remained around the roots. There are however, limitations on how to grow plants hydroponically. Coarse gravel was used in this case to stabilise the plants and to maximise flow around the roots. Since the gravel was used as a standard approach the results among the different species are directly comparable.

Finally, it is impossible to be entirely confident that hydroponic plants received adequate oxygenation, the results however, were sensible in terms of the classic responses exhibited by plants in response to drought stress. The only exception perhaps being the results for the osmotic potential at full turgor in PEG stressed plants (see Chapter 5).

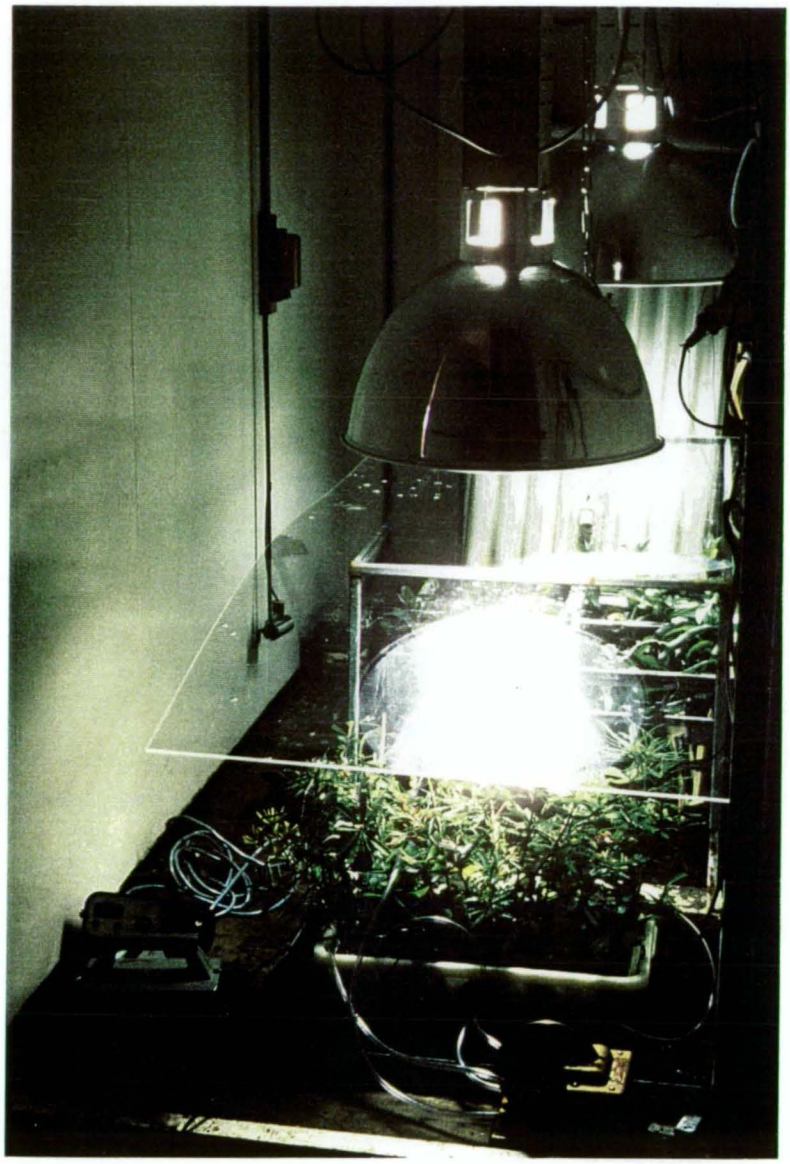

Figure 4.2. An example of the hydroponic system used for the frost and drought experiments.

Frosting Treatment Method

The techniques available for frost studies range from the simple to the very complex. Indeed, a lot of work on plant frost tolerance has been done at a cellular, physiological, biochemical and molecular level. These studies are primarily concerned with identifying the actual physiological and biochemical mechanisms used by plants to survive cold stress. The electrical conductivity method used in the following experiments provides a simple, comparative measure of species frost tolerance.

The electrical conductivity method for the measurement of plant frost tolerance was first used by Dexter *et al.* (1930). This method is based on the principle that intracellular freezing results in bio-membrane damage. As the frozen plant tissue thaws, electrolyte leakage from the cell vacuole occurs. The magnitude of leakage can be detected using an electrical conductivity meter.

For each frost experiment a specific number of leaf discs were taken per plant using a hole puncher. After cutting, the leaf discs were placed in a glass vial with a drop of de-ionised water and a few crystals of AgI to ensure ice crystal formation and thus prevent the supercooling of samples during the frost treatment. Each disc sample was then artificially frosted in a frost cabinet (height 750 mm, width 500 mm, depth 520 mm) at the following temperatures: -4, -7, -9, -12, and -15°C. All samples for the frost treatment were randomly placed on an aluminium tray located on the floor of the cabinet. Samples were placed on a tray of aluminium because the high thermal conductivity of this metal results in only minor temperature fluctuations between the vials (Hallam and Tibbits 1988). Please note that the specific details of each frost experiment will be described in the relevant chapters.

The same frost cabinet used by Hallam and Tibbits (1988), Read and Hill (1988, 1989) and Read and Hope (1989) was used for this study. A freezing coil is situated near the cabinet floor and an air mixing fan is located on the roof. Refrigerant is directed either through the coil within the chamber (cooling on) or through an external coil (cooling off) by means of two solenoid valves (Hallam and Tibbits 1988). The required cooling rate of the cabinet must be selected and is controlled electronically to within 0.1-0.2 °C. The

cabinet temperature is sensed by a transistor and this information is fed into an electronic circuit which controls the operation of the solenoid valves during cooling. For a more detailed description of the cabinet see Hallam and Tibbits (1988). Information regarding the temperature of the frost cabinet was recorded on a Mace FBQ 100 lab recorder.

To create a frost within the cabinet the temperature was rapidly lowered at 1.0 °C min^{-1} until 2.0°C had been reached, after which the cooling rate was reduced to 0.2°C min^{-1} (Read and Hope 1989) where it remained until the test temperature had been reached. The samples were held at the required temperature for 45 minutes. At the end of the treatment, the cabinet door was left slightly ajar until the frost cabinet temperature had warmed to 2°C. This temperature was determined from a thermometer placed in the cabinet after opening. The specimens were then removed from the cabinet and left to thaw at room temperature for 60 minutes. The length of time it took for each treatment to warm to 2°C after testing varied according to the test temperature. Read (1985) however, indicates that the rate of warming post frosting does not have a significant effect on tissue damage unless water is added to the specimens immediately post treatment.

After the frost treatment, 10 ml of de-ionised water was added to each glass vial. The vials were then sealed and shaken for 24 hours. At the end of this time, the conductivity of the solution was measured with a Wissenschaftlich - Technische Werkstatten (WTW), temperature corrected conductivity meter. Finally, the specimens were boiled in a microwave oven with the lids placed loosely on the lip of each vial to minimise evaporation. After boiling, the vials were topped up with de-ionised water to maintain the standard content of 10 ml. Hallam and Tibbits (1988) and Read and Hope (1989) have both indicated that electrolyte leakage which results from heat damaged tissue is the same as that which results from frost damaged tissue. Differential heating occurred in the microwave oven, so all samples were closely observed to ensure they reached boiling point. The vials were then placed on a shaker to agitate for a further 24 hours and the conductivity was re-measured. A control was also set up in which the leaf discs were immediately placed in glass vials with 10 ml of distilled water. The vials were treated as above, but were not exposed to freezing temperatures.

It values (tissue damage) were calculated for each control and each plant at each temperature from the following formula:
It = 100 x *(Rt-Ro)/(1-Ro)*

Where *Rt = Lt/Lk* and *Ro = Lo/Ld* and,

Where *Lt*=conductivity after freezing
Lk=conductivity after boiling
Lo=conductivity for unfrozen control and,
Ld=conductivity for control after boiling.

The *It* values were transformed to give *T50s* (i.e. the temperature at which 50% tissue damage occurs) using a probit transformation in the SAS statistical package, (SAS Institute Inc. 1988), see page ~~49~~ 56.

This technique has the following advantages over the artificial frosting of whole plants:

(1). the use of leaf discs rather than whole plants means that horizontal temperature gradients in the frost cabinet have a minimal effect on the results (Read 1985),
(2). the experiment is non-destructive to the plant as a whole so the same plants can be repeatedly sampled,
(3). there is no limitation on the size of the plants that can be tested because leaf discs are used (Hallam and Tibbits 1988) and,
(4). artificial effects on roots in a small soil space are avoided.

However, like most techniques used in ecophyisological studies, there are always weaknesses and potential sources of error. Thus, it is important to be aware:
(1). of the importance of using standard sized leaf discs for all experiments. Different sized leaf discs of the same material have been observed to produce different levels of tissue damage when frosted at the same temperatures (Read 1985, Hallam and Tibbits 1988),
(2). there is a certain amount of electrolyte leakage from the cut perimeter of all leaf discs. To avoid any error associated with this factor, Flint *et al.* (1967) developed the index of injury unit *(It).* The *It* value is weighted by the control value, so that *It* = 0 in unfrozen tissue and *It* = 100 in fully damaged tissue.

Hence, it eliminates differences due to changes (and inherent differences) in leakiness of the unfrozen (control) tissues and allows direct comparison to be made among the species (Read and Hill 1988).

(3). that Harwood (1981), Read (1985) and Hallam and Tibbits (1988) have noticed that faster rates of cooling result in significantly greater frost damage for a set temperature than slower rates. Thus, it is important to have a standard cooling rate for all experiments.

(4). that Read (1985) indicated that the rate of warming post-frosting does not have a significant effect on tissue damage unless water is added immediately post treatment, in which case the tissue damage will be greater than if the specimens were thawed more slowly.

(5). that Read (1985) suggested that exposure of leaf discs to periods of frost for longer than 30 minutes does not significantly affect tissue damage.

Water Relations Experiments

It is important to consider as many aspects of a species' water relations as possible when attempting to determine the drought tolerance and past and present distributions of certain species. This is because the capacity of a plant to resist or tolerate drought stress results from the integration of a wide range of adaptive characteristics and mechanisms (Jones and Turner 1980, Turner 1979). For this study the Scholander style pressure bomb was used to generate pressure volume curves for the *Banksia* species examined. These curves provided the following information:

(1). Osmotic potential at full turgor *(π_O)* (MPa)
(2). Relative water content of the apoplast*(R*a)* (units range 0-1)
(3). Bulk elastic modulus *(ε)* at near full turgor (MPa)
(4). %Relative water content at turgor loss (units range 0-100%)
(5). Water potential at turgor loss (MPa)

The pressure bomb was first designed by Scholander, Hammel, and their colleagues at the Scripps Institute of Oceanography in 1965 (Ritchie and Hinckley 1975). The pressure bomb is reliable, easy to use and data can be rapidly obtained (Ritchie and Hinckley 1975). Over the years, the pressure bomb has been favoured for many field and lab based water relations studies. It has been a popular choice for field studies, particularly for the determination of dawn to dusk water potentials (Dodd and Bell 1993a, 1993b). The bomb has also been popularly used in the production of pressure volume curves both in the field and lab, for example, by Roberts *et al.* (1980), Mooney and Davis (1986), Ranney *et al.* (1991), Vance and Zaerr (1991) and Andersen and McLaughlin (1991).

In spite of the bomb's popularity, a recent study using both pressure probes and bombs to determine the water relations of *Nicotiana rustica* and *Plantago* sp. suggests that more negative xylem water potentials are obtained from pressure bombs than is reality (Balling and Zimmermann 1990). Balling and Zimmermann (1990) maintain that the bomb does not indicate the true tension in the xylem vessels of *Nicotiana* and *Plantago* spp. Balling and Zimmermann (1990) believe that xylem water potentials can be more accurately measured with pressure probes. Pressure probes can be directly

inserted into the xylem vessels and the cells surrounding the probe are thought to form a natural seal. Balling and Zimmermann (1990) are strong advocates of the pressure probe, they do stress that more work must be done with probes before the xylem Ψ results obtained from *Nicotiana* and *Plantago* spp. can be accepted with certainty.

Balling and Zimmermann's (1990) experiments on *Nicotiana* and *Plantago* spp. indicated that the average difference between the xylem tension obtained from the probe and bomb is 0.25 MPa in well watered plants. They thus propose that the bomb method may still be useful in the measurement of the xylem tension of taller plants and trees, where presumably higher tensions are expected than in *Nicotiana* and *Plantago* spp. It has also been suggested by Zimmermann and Brown (1980) that the real value of the pressure bomb technique lies more in comparative results than in an accurate determination of the absolute values of the tension in the xylem (Zimmermann and Brown 1980).

Additionally, Tyree (1981) looked at the relationship between bulk elastic modulus (ε) as derived from the pressure bomb and the individual cellular ε of leaves as measured by the pressure probe technique. An analysis of the sources of error resulting from the evaluation of ε by the pressure bomb reveals elasticity can be calculated within an error of 10%. This error is similar to the error in ε measured by the pressure probe technique (Tyree 1981).

An obvious weakness in the experiments of Balling and Zimmermann (1990) is that they dealt only with high pressures, e.g. the most negative pressure they recorded was -0.4 MPa. Additionally, it has been suggested that the difference in xylem tension values between the pressure probe and the pressure bomb may be due to the osmotic components of the xylem (Smith and Luttge 1985, Smith and Murphy 1989). For example, Smith and Murphy found that pressure probe and bomb xylem tension measurements were comparable provided the π_O of the xylem was considered.

Further concerns over pressure bombs for water relations have been raised by Puritch and Turner (1973), as cited in Ritchie and Hinckley (1975). They suggested that the intra-chamber temperature rise which accompanies chamber pressurisation may result in negatively deflated π_O. This is

because osmotic potential and temperature are directly related, e.g. osmotic potential = $C_S RT$, where C_S is the molar concentration of the solute, R the gas constant and T the Kelvin temperature (Slatyer 1967). The magnitude of the rise in chamber temperature is directly related to the rate of pressurisation, with faster rates producing a greater intra-chamber temperature increase than slower rates (Ritchie and Hinckley 1975). Ritchie and Hinckley (1975) have indicated that increased solute concentration may also result from temperature induced, elevated metabolic rates. Additionally, they have suggested that alterations in turgor and matric potentials may also occur in response to pressurisation.

It is clear that there is some doubt about the validity of the use of Scholander style pressure bombs in water relations studies. More work must be done with pressure probes before they can be widely accepted as a water relations technique, especially since the Scholander style pressure bomb has been in popular use for almost 40 years. The pressure bomb was used to perform the water relations experiments in the following chapters because of its usefulness for comparative purposes.

Pressure Volume Curve Analysis

Prior to performing the actual pressure volume curve analyses procedure *(PVC)*, shoots were excised from the terminal ends of branches under distilled water to prevent the entry of air into the xylem cavity. Entry of air into the xylem may contribute to false end-point readings from the pressure bomb gauge (Turner and Begg 1981). False end-points have been observed as bubbling at the cut surface of shoots and is due to the passage of air out of the xylem carrying the xylem content with it (Klepper and Ceccato 1968). Bubbling eventually gives way to fluid exudation i.e. the true end-point, however, recognition of this tends to require experience (Klepper and Ceccato 1968). In *PVC* analysis, the cutting of stems underwater is designed to reduce the risk of air entering into the xylem and creating false end-points on pressurisation. For this study, after excision, the shoots were labelled and the cut ends were placed in distilled water (Mooney and Davis 1986). The shoots were covered in a plastic bag and placed in a cool, dark room at 5°C. The plastic covering and cool, dark room were designed to retard water loss through transpiration and so facilitate the achievement of full turgidity by the shoots. Davie *et al.* (1993) maintain that most plant species attain full turgidity within a minimum of four hours.

The next day, the shoots were blotted dry and the turgid weight and corresponding water potentials (Ψ) were obtained. To obtain the Ψ, shoots were placed so that the foliage was concealed within the pressure bomb chamber, thus the only visible part of the specimen was the cut end of the shoot which was extruded through the silicon washer. The chamber was then pressurised with nitrogen gas from a storage cylinder at the approximate rate of 0.025 MPa sec^{-1} (Turner and Begg 1981). The pressure at which the sap appeared at the surface of the cut end of the shoot, was recorded as the end-point Ψ of the plant. Turner and Begg (1981) report that care must be taken with the rate at which the chamber is pressurised since rapid rates lead to more negative Ψ than slow rates. This is because, as explained earlier, faster rates of pressurisation result in increased intracellular solute concentration (Ritchie and Hinckley 1975). After pressurisation, the gas in the chamber was released slowly through a valve. A hand held lens with a light attached was used to magnify the specimen so that the sap end-point could be easily detected. The shoots were then placed on a gauze rack to air dry, the fresh weight and Ψ were periodically

recorded until after the point of tissue turgor loss. After turgor loss, the shoots were oven dried at 60°C for one week and the true dry weights of each shoot were subsequently measured (See page 55 for discussion of the temperature used to dry the discs). The true dry weight values were then used to re-draw the *PVC* curves. The true π_0, R^*a, ε, water potential and relative water content at turgor loss were determined from the re-drawn curves.

Throughout the experiment, the reciprocal of the water potential $(1/\Psi)$ was plotted against the corresponding *RWC* for each specimen. The dry weight of each *Banksia* species was estimated prior to the *PVC* analyses so that an estimate of the true *RWC* could be calculated during the experiment.

To obtain an estimate of the dry weights prior to the *PVC* analyses, the following procedure was followed. Five plants from each species and/or population were randomly selected. One shoot was randomly selected from each plant. The stem of each shoot was placed in a container of de-ionised water for 24 hours in order for the shoots to attain full turgidity. At the end of this period, the turgid weight of each shoot was recorded. The shoots were then oven dried at 60°C for approximately one week. At this temperature, one week was usually long enough for the weight of the shoots to have stabilised thereby indicating that the shoot tissues were fully dry. Once dry, the shoot dry weights were recorded. The following formula was then used to determine the % of weight that the dry component of each shoot had contributed to the turgid weigh of the shoot:

$$\%DW = DW/TW \times 100/1$$

where *DW*=dry weight and *TW*=turgid weight.

The mean of the *%DW* value calculated from the above formula for each species and/or population was then used as an estimate of the dry weight of each turgid shoot for the *PVC* analyses.

$$DW_e = TW/1 \times DW/100$$

where DW_e= mean dry weight of experimental sample. and mean dry weight of preliminary sample.

Using the dry weight estimate, *RWCs* were obtained for each shoot through the entire *PVC* analyses using this formula :

%RWC=100 x *(FW-DW)/(TW-DW),*

where *RWC*=relative water content, *FW*=fresh weight, *TW*=turgid weight and *DW*=dry weight (Slatyer 1967).

The *PVC* analyses were continued until the regression of the linear relationship between the reciprocal of the balance pressure *(1/Ψ)* and the *RWC* content yielded a correlation coefficient of > 0.92 or better for at least five points along each graph. The graphs enabled the point of turgor loss to be visually detected, i.e. where the graphs became linear.

Pre-dawn Water Potentials

Pre-dawn Ψ_s are a common part of many experiments investigating field levels of drought stress in plant communities e.g. (Dodd and Bell 1993a, 1993b, Tunstall and Connor 1975). Pre-dawn Ψ_s provide a check on the base level of the Ψ of individual members within a plant community. In this way it is possible to determine whether the individuals chosen for an experiment are a true representation of the community (Tunstall and Connor 1975). Furthermore, pre-dawn Ψ_s provide an indication of the level of drought stress a plant is under at the least stressful time of the day, e.g. when it is dark and water is not being lost from the plant through evapo-transpiration. Pre-dawn Ψ_s also expose any differences in the soil microhabitat of the plants being examined e.g. any significant differences observed in the pre-dawn Ψ of individual plants within a community may be related to differences in their soil microhabitats.

The Scholander style pressure bomb is also used to obtain pre-dawn Ψ_s. Consequently, the same potential problems associated with this equipment will also apply to the calculation of pre-dawn Ψ_s.

As the name implies, these Ψ_s are obtained pre-dawn. It is important therefore to have a good source of light. For this study, shoots of adult foliage from a south facing aspect were removed with secateurs and placed in the pressure bomb, within seconds the Ψ were recorded. The time before

dawn at which each population was sampled varied between 2:30-5:30 am. The time was dependent on the length of time it took to travel to and carry out the tests at each site. Refer to page 49, paragraph 2, lines 2-15 for information on equipment usage to obtain the Ψ.

Desiccation Treatment

It has been observed by Blum and Ebercon (1981), Read 1985, Martin *et al.* (1987) and Tan and Blake (1993) that the relative leakage of electrolytes from cells or tissues which have been exposed to water stress can be used to indicate the dehydration tolerance of species. Read (1985), Martin *et al.* (1987) and Tan and Blake (1993) have all observed that drought tolerant plants exposed to dehydration stress, leak fewer electrolytes than less drought tolerant plants. In other words, the greater the cell membrane integrity of a plant the more drought tolerant it is likely to be. Although the techniques used by the above researchers to dehydrate their plants differed, the common link is that they all used conductivity meters to determine tissue damage by electrolyte leakage. The progressive desiccation of plant tissue ultimately results in damage to the protoplasmic structures of cells, especially the bio-membranes (Larcher 1980).

The desiccation method used for this study is a modification by Read (1985) of the electrical conductivity method first used by Dexter *et al.* (1930) to determine frost damage. The value of this technique is that it is simple to carry out although it is labour intensive. The *T50* values obtained should not be viewed as precise quantitative values but rather as a convenient reference point from which to compare the desiccation tolerances of the different populations. The main value of the *T50s* is that they provide a means of comparing tolerances among species and populations while giving an indication of species tolerance to stress.

The principles behind the desiccation and frost experiments are very similar. In both cases, tissue damage is reflected by the magnitude of electrolyte leakage from the damaged protoplasmic structures and is measured with a conductivity meter.

For this experiment, two 6 mm diameter leaf discs were taken from each of two leaves per fully turgid shoot and weighed as a unit (i.e. four discs in total from each shoot = 1 unit). The initial weight from each leaf disc unit was recorded as the turgid weight. To prevent the leaf discs drying out before use, the leaf disc units were placed abaxial surface down into segmented petri-dishes with a filter paper insert which had been dampened with de-

ionised water. Lids were placed on the petri-dishes and the specimens were placed in a cool, dark room at 5°C over night.

The next day, each unit of four discs was placed into a micro-desiccator to desiccate.

The micro-desiccator was made from a pyrex casserole dish. A rubber ring was placed around the lid's edge to provide a seal. A metal, circular frame with four legs was covered with a piece of fibreglass flyscreen to form a stage for the desiccation of each leaf disc unit. The leaf disc units were laid abaxial surface down upon this stage, 8 mm above the surface of concentrated (98.07%) H_2SO_4. The effect of the acid was to absorb the moisture in the air of the desiccating chamber above the acid, thereby promoting dehydration of the leaf discs. The rate of desiccation was not critical to this study, it was more important that all leaf discs were dehydrated under the same conditions. The objective of this experiment was to use a method to promote tissue dehydration to enable a reliable correlation between tissue *RWC* and damage. In this case, the *Banksia* tissues were extremely desiccation tolerant even at low relative water contents in spite of their rapid desiccation treatment (see Chapter 9).

The wire stage was divided into enough segments so that all the leaf disc units from one site could be desiccated at the same time. It was impossible to desiccate any more than one site at a time because desiccation occurred quite rapidly.

During desiccation, the leaf disc units were removed from the desiccator periodically and the fresh weights were obtained for each of the individual leaf disc units. By doing this, a range of *RWCs* over a range of desiccation injuries was obtained. After desiccation, the leaf units were placed into glass vials with 10 ml of de-ionised water. The specimens were shaken for 24 hours. At the end of 24 hours of shaking, the conductivity of the fluid bathing the leaf discs in the glass vials was determined with a WTW conductivity meter. The specimens were then microwaved to boiling point to ensure maximum tissue damage and hence electrolyte leakage. Lids were placed on top of vials to minimise evaporation during this process (Hallam and Tibbits 1988). The vials were topped up after boiling to 10 ml. After boiling, specimens were shaken for a further 24 hours, and the electrical conductivity

was measured. The values for conductivity were then substituted into the same formula used to determine the *It* values for the frost experiments, see below:

It=100 x(Rt-Ro)/(1-Ro)

Where *Rt=Lt/Lk* and *Ro=Lo/Ld* and,

Where *Lt*=conductivity after dehydration
Lk=conductivity after boiling
Lo=conductivity for undesiccated control and,
Ld=conductivity for control after boiling.

After the final conductivity readings, the leaf disc units were placed in an oven at 60°C to dry. The weight of the leaf discs was checked periodically. The temperature at which the leaves are dried is not critical, since the objective of drying is to leave the specimens in the oven until a constant weight has been reached. Once the weight had stabilised, the dry weight was recorded for each sample of the leaf discs. For comparison, after PVC analyses Davie *et al.* (1993) dried leaf material at 70°C in an oven until the material had reached a constant weight while Losch *et al.* (1982) obtained the dry weights of leaves by drying them in an oven at 105°C.

The following formula was used to determine the *% RWC* for each leaf disc unit at each level of desiccation:

%RWC=100 x (FW - DW)/(TW - DW),

Where *RWC*=relative water content, *FW*=fresh weight, *TW*=turgid weight, and *DW*=dry weight (Slatyer 1967).

A control was set up where leaf discs were not desiccated but were otherwise put through the same procedure as described above. The data obtained from the controls were substituted into the above equation to help determine the *It* values.

The desiccation *It* data values were transformed to give *T50s* (i.e. *RWC* at which 50% tissue damage occurred) using a probit transformation in the SAS statistical package (SAS Institute Inc. 1988), see below.

Data Analysis

Probit Transformation

Probit transformations have been used in the analysis of biological experiments, particularly those that consist of a stimulus and a subject (Finney 1947). When damage *(It)* is plotted against its stimulus e.g. temperature/dehydration, the response effectively resembles a sigmoid curve, i.e. there is minimal tissue damage at low stress levels, with damage gradually increasing until a peak has been reached at which the curve plateaus off.

From the probit transformed frost and desiccation *It* data it was possible to extrapolate the *T50* values (i.e. the temperature or relative water content at which each plant examined succumbed to 50% tissue damage). *T50* values obtained in this way are more accurate than those obtained by estimation directly off the graphs.

CHAPTER 5. WATER RELATIONS EXPERIMENTS

Introduction

Water is generally considered to be the predominant factor limiting carbon gain and primary production over a multitude of ecosystems (Lajtha and Gertz 1993).

Many species of plant with similar capacity to survive drought often do so using completely different mechanisms. For example, some species are drought avoiders, i.e. where the rapid onset of stomatal closure in response to water stress results in the maintenance of high *RWC* and Ψ. For example, Withers (1978) considers that the desiccation avoidance of *Casuarina littoralis* is facilitated by its capacity for the efficient reduction of stomatal and cuticular transpiration. Other species survive drought because they possess extensive root systems which enable them to tap into the water table i.e. have a phreatophytic habit. Sinclair (1980) suggests that the superior drought tolerance of *Eucalyptus fasiculosa* and *E. leucoxylon* over *E. obliqua* is probably related to the superior depth, extent and effectiveness of their rooting systems. Other species actively accumulate solutes, i.e. osmotic adjustment (see Rhizopoulous and Mitrakos 1990, Ranney *et al.* 1991, Davie *et al.* 1993 and Fan *et al.* 1994) to produce plant tissue Ψ which are more negative than Ψ of the soil, thereby facilitating the movement of water from the soil into the plant. Indeed, the above scenarios are only a small sample of the possible mechanisms used by different species to survive drought. Thus, when looking at the comparative drought tolerance of species it is important to consider as many aspects of tissue water relations as possible, to ensure that important aspects of species drought survival are not overlooked.

The following *PVC* experiments were an attempt to highlight any physiological weakness in the drought survival capacity of the Pleistocene fossil species, *B. kingii* and *B. strahanensis*, which may have contributed to their extinction from Tasmania. For the following experiments, the drought tolerance of eight Tasmanian populations of the very successful *B. marginata* were used as a benchmark from which to compare the drought tolerance of

the less successful, closest living relatives of the fossil species, i.e. *B. canei*, *B. saxicola* and *B. spinulosa* var. *cunninghamii*.

Methods

A minimum of eight plants, 12-15 months old, were randomly selected from glasshouse grown specimens (Table 5.1).

Table 5.1 List of *Banksia* species examined for drought tolerance.

Species	State	Site
Banksia canei	Victoria	Omeo
Banksia saxicola	Victoria	Mount William
Banksia saxicola	Victoria	Wilsons Promontory
Banksia spinulosa var. *cunninghamii*	Victoria	Wilsons Promontory
Banksia marginata	Tasmania	Melaleuca
Banksia marginata	Tasmania	Birches Inlet
Banksia marginata	Tasmania	Snug Plains
Banksia marginata	Tasmania	The Gardens
Banksia marginata	Tasmania	Cape Hauy
Banksia marginata	Tasmania	Cape Tourville
Banksia marginata	Tasmania	Mount Wellington
Banksia marginata	Tasmania	The Blow Hole

Refer to Fig. 4.1 for the sites of origination of the glasshouse grown seedlings of *B. marginata.*

The hydroponic experiments were performed on plants which varied in age, i.e. between 12-15 months as stated previously.

The plants were grown hydroponically in a growth cabinet three weeks prior to *PVC* analysis, under a 12 hour photo-period at constant night and day temperatures of 10°C and 12°C respectively. Plants were kept cool to facilitate the dissolution of O_2 in the hydroponic medium.

The hydroponic method used for this experiment is a modification of the method used by Zwaizek and Blake (1989).

At the beginning of the experiment, the plants were divided into two groups,
(1). the control and,
(2). the drought treated group.

The controls were given no drought stress and were grown in half strength Hoagland's nutrient solution for a total of three weeks before the PVC analyses.

The drought treated group were also grown for a total of three weeks before the *PVC* analyses. Zwaizek and Blake (1989) used a hydroponic system and PEG 3350 to drought pine seedlings. The methodology of Zwaizek and Blake (1989) was modified. For this experiment certain aspects of same time frame and PEG concentrations presented by Zwaizek and Blake (1989) were used.

The drought treated plants for the first two weeks of the three week total were grown in Hoagland's nutrient solution to give them time to acclimate to the new growth medium prior to the drought stress treatment (Zwaizek and Blake 1989). At the beginning of the third week, drought stress was imposed by adding PEG 3350 to the bathing medium of the plants at the concentration of 150g L^{-1} to produce an osmotic stress of -0.5 MPa (Zwaizek and Blake 1989). The plants were kept at -0.5 MPa for four days, after which PEG 3350 was added at a concentration of 100g L^{-1} to decrease the π_O of the

solution to -1.0 MPa for the next three days (Zwaizek and Blake 1989). In total, the period of drought stress did not exceed seven days.

Similar to Zwaizek and Blake (1989), no toxicity was observed in the plants treated with PEG 3350. After three weeks in hydroponics, both the control and drought stressed plants were sampled for *PVC* analysis. Refer to Chapter 4 for details of the *PVC* technique (if necessary).

It could be argued that seven days of drought stress would not have been adequate to obtain significant results. However, unlike traditional drought stress experiments where stress is induced by witholding water, the hydroponic experiments used in this study can be carried out over a shorter period because there is not the time lag as in the pot experiments i.e. waiting for the soil water content to decrease to a certain level.

There is no typical length of time over which plants are droughted by experimenters. The length of time varies case by case and should be dependent on the aims and objectives of the study. Where plants are droughted by withholding water, factors such as plant size (root and shoot ratio), pot size and soil volume and type should be considered. Examples of the different time frames used to induce drought stress include: three and four weeks for *Eucalyptus viminalis* seedlings which were "hardened off outside" (Ladiges 1974, 1975) respectively, Withers (1978) droughted *Eucalyptus* and *Casuarina* seedlings by witholding water for a maximum of nine and twelve days respectively, Zwaizek and Blake (1989) used different concentrations of PEG to gradually drought black spruce over a seven day period, Premachandra *et al.* (1993) tested the cell membrane stability of orchard grass by immersing pieces of leaf in PEG solution for 24 hours, Fan and Blake (1994) droughted seedlings of eucalypt and pine species by witholding water over an eight day period and Bachelard (1986) examined the effect of drought stress of eucalypt seedlings which had been been gradually hardened over a period of many weeks.

Data Analysis

The program 'TISWAT.BAS' (Davie *et al.* 1993) was used to calculate the osmotic potential of fully turgid plants (π_o), the relative water content at

turgor loss, water potential at turgor loss, relative water content of the apoplast *(R*a)* and bulk elastic modulus at near full turgor *(ε)*.

As mentioned previously (Chapter 4), throughout the *PVC* experiments, the *1/Ψ* was plotted against the corresponding *RWC* for each shoot. As the curve of the graphs became linear it was an indication that the plant tissues had reached and passed the point of turgor loss. Within the TISWAT program, regression analyses were performed across the linear portion of the graph and then extrapolated to the Y axis to determine the π_O at full turgor for each seedling. For example, in consideration of the Ψ equation, i.e. where plant $\Psi = P + \pi + \tau$ and where matric potential *(τ)* generally contributes little to the overall Ψ of most plants, except those which are highly sclerophyllous, the removal of turgor potential *(P)* from the above equation when a shoot reaches turgor loss will result in a value for the π_{tlp} of the plant.

However, in acknowledgment of the sclerophyllous nature of the *Banksia* seedlings studied, the significance of the contribution of τ to the overall Ψ remains unknown. Regardless however, the *PVC* technique is at least suitable for obtaining comparative trends in the π_O of these species rather than perhaps producing precise quantitative values. As an example of the effect of τ, Larcher (1980) maintains that the surface forces of the cell walls of plants can hold water with a pressure of 1.5-15 MPa. The pressure will depend on the density with which the cell wall fibres are packed (Larcher 1980).

As just indicated, estimates for π_O are obtained from the intercept of linear regressions with the Y axis. By contrast, estimates for *R*a* are taken from the linear regression point of interception with the X axis. This intercept separates the *RWC* of the symplast from the apoplast. Pressure volume curve analyses assume there is no change in the *R*a* of the tissue during pressurisation (Davie *et al.* 1993). Estimations of *R*a* involve considerably more extrapolation than estimates of π_O and are therefore less accurate (Davie *et al.* 1993).

The point where the extrapolated line meets the x axi
when the symplastic water has decreased to zero i.e.

The capacity of plant tissue to adjust in volume in response to drought stress is measured as the bulk elastic modulus *(ε)* i.e. the average change in turgor pressure *(Δ P)* for a fractional change in relative water content *(Δ RWC)* (Blake and Tschaplinksi 1992):

$\varepsilon = (\Delta P/\Delta RWC) X RWC$ (Blake and Tschaplinksi 1992).
In this case, lower values of ε are characteristic of tissues more elastic than tissues of higher values of ε (Blake and Tschaplinksi 1992).

Statistical Analysis

A two way factorial ANOVA, was performed using SAS (SAS Institute Inc. 1988) to determine whether there was a significant difference, a) between the different treatments for osmotic potential at full turgor, apoplastic water content, bulk modulus of elasticity, water potential and relative water content at turgor loss, b) among the species and c) a species*treatment interaction.

A one way ANOVA was carried with *a posteriori* comparisons using Tukey's method to determine whether there were significant differences among the different species for the treatments (hardened and unhardened).

Results

Bulk Elastic Modulus

Figure 5.1 demonstrates a reduction in the ε value of the droughted specimens compared to the controls for all populations of the *Banksia* species. Please note, the greater the cell wall elasticity, the smaller the ε value is in MPa. Analysis of variance (see Appendix 1) indicates significant differences ($P < 0.05$) between the two treatments for ε. Table 5.2 demonstrates the magnitude of adjustment in ε between the two treatments. This table is an aid to the interpretation of Figure 5.1.

Tukey's test (see Appendix 1) indicates that the drought treated Mount Wellington *B. marginata* population and *B. canei* had significantly lower values of ε than all other populations of *Banksia* except populations from the Blowhole and Snug. Of the drought treated populations, *B. spinulosa* var. *cunninghamii* and *B. saxicola* from Wilsons Promontory had the highest ε values.

Control *B. spinulosa* var. *cunninghamii* seedlings were significantly different ($P < 0.05$) from seedlings of *B. marginata* from the Mount Wellington,

(Birches Inlet), The Blow Hole and The Gardens populations. The *B. spinulosa* var. *cunninghamii* seedlings had significantly ($P < 0.05$) higher values of ε than those populations of *B. marginata*. Note that the Birches Inlet population is in brackets because it was inadvertently omitted from the statistical analysis. Please note however, that the result for this population have been graphed.

The populations and/or species with the most elastic cell walls were the PEG stressed *B. canei* from Omeo and *B. marginata* from Mount Wellington, Snug and the Blowhole.

Analysis of variance indicates significant difference ($P < 0.05$) in the bulk elastic modulus among populations of *B. marginata* from Tasmania for the drought treatment i.e. with Mount Wellington being significantly different ($P < 0.05$) from all other populations of *B. marginata* except populations from Snug and the Blowhole (see Tukey's test in the Appendix 1). There were no significant differences ($P > 0.05$) among the populations of *B. marginata* for the control treatment (see Tukey's test in Appendix 1).

Elastic adjustments result from structural modifications in the cell wall which increase cell wall ε thereby improving a species drought tolerance by facilitating tissue shrinkage during dehydration. Joly and Zaerr (1987) have suggested that elastic shrinkage rather than osmotic adjustment may be of more importance for drought resistance in repeatedly stressed woody plants.

Apoplastic Water Content

Analysis of variance (Appendix 1) indicates a significant difference between the two treatments in terms of the *R*a*. Figure 5.2 demonstrates that all populations of *B. marginata* except the population from Birches Inlet increased their *R*a* when droughted. Of the Victorian species, drought stressed *B. spinulosa* var. *cunninghami* was the only species which increases its *R*a*. Drought stressed *B. canei* and *B. saxicola* from Mount William and Wilsons Promontory underwent a decrease in *R*a*.

Analysis of variance indicates no significant difference ($P > 0.05$) among any of the *Banksia* species examined for the drought treatment. The control seedlings of *B. canei* and *B. saxicola* from Wilsons Promontory were not

significantly different ($P > 0.05$) from any of the populations of *B. marginata* except from the Blowhole, Melaleuca and Mount Wellington. In this case, both species had significantly higher *R*a* than *B. marginata* from the Blow Hole, Melaleuca and Mount Wellington.

Figure 5.2 indicates that the PEG stressed species with the highest *R*a* were *B. marginata* from Mount Wellington, followed by the Snug, Cape Tourville and Blowhole populations.

Figure 5.2 indicates the control species with the highest *R*a* were *B. saxicola* from Wilsons Promontory and *B. canei*. Although, the *R*a* of these species decreased in response to drought stress.

Interpopulation physiological variability in *B. marginata* is highlighted by the analysis of variance which indicates significant differences ($P < 0.05$) in the *R*a* among the control populations of *B. marginata* from Tasmania. The Birches Inlet population of *B. marginata* differed from the Blow Hole, Melaleuca and Mount Wellington (Fig. 5.2). The Cape Hauy population differed significantly ($P < 0.05$) from the Mount Wellington population.

High *R*a* has been observed in plants which have been pre-conditioned to water stress, protecting living cells from damage from sudden water loss during subsequent dehydration (Blake and Tschaplinski 1992).

Osmotic Potential

Analysis of variance indicates a significant difference ($P < 0.05$) between the treatments for the osmotic potential at full turgor. Figure 5.3 demonstrates that in most cases the control seedlings have more negative π_O at full turgor than the drought stressed seedlings. *Banksia spinulosa* var. *cunninghami* and *B. saxicola* from Wilsons Promontory were the only two species in which there was little discernable difference between the π_O of the controls and the PEG stressed plants. Table 5.3 demonstrates the magnitude of adjustment in π_O between the two treatments. This table is an aid to the interpretation of Figure 5.3.

The above results are unusual in water relations studies. Most often, the π_O in drought stressed plants will either change very little or will become more negative as the plant adjusts its π_O to be lower than the soil Ψ thus facilitating the movement of water from the soil into the roots. Recently however, a phenomenon called osmotic de-adjustment has been described, where the π_O of some species has been reported to become less negative after drought stress when compared to their controls (Blake and Tschaplinski 1992). Interestingly, Bowman and Roberts (1985) have also found some chaparral shrubs produced artificially higher π_O upon re-hydration after a drought.

Tukey's test (Appendix 1) indicates no significant difference ($P > 0.05$) between the π_O values of populations of *B. marginata* and the π_O of *B. canei* and *B. saxicola* from Wilsons Promontory for the PEG stressed or control treatments. The control seedlings of *B. saxicola* from Mount William were significantly ($P < 0.05$) different from the Mount Wellington, The Gardens and (Birches Inlet) populations. Control seedlings of *B. spinulosa* var. *cunninghamii* had significantly ($P < 0.05$) more negative π_O than populations of *B. marginata* from Snug, Melaleuca, Blow Hole, (Birches Inlet), The Gardens and Mount Wellington. The π_O of the PEG stressed *B. spinulosa* var. *cunninghamii* seedlings was significantly ($P < 0.05$) more negative than the π_O of all of the populations of *B. marginata*, except those from Cape Tourville, Cape Hauy and (Birches Inlet).

The control species with the most negative π_O at full turgor were the fossil relative species, *B. spinulosa* var. *cunninghamii* at -2.157 MPa and *B. saxicola* from Mount William at -1.905 MPa.

The PEG stressed species with the most negative π_O at full turgor were the the fossil relative species, *B. spinulosa* var. *cunninghamii* at -2.1414 MPa, followed by *B. saxicola* from Wilsons Promontory at -1.5053 MPa.

Analysis of variance indicates no significant difference ($P > 0.05$) in the π_O among the populations of *B. marginata* examined for either the PEG or control treatments.

Relative water content at turgor loss

Analysis of variance indicates a significant difference ($P < 0.05$) between treatments for the relative water contents at turgor loss. Figure 5.4 demonstrates a reduction in the relative water content at turgor loss for all populations of PEG stressed *Banksia* when compared to the controls with the exception of *B. marginata* from Mount Wellington.

Tukey's test indicates that the following species: *B. canei* and *B. saxicola* from Wilsons Promontory and Mount William respectively and *B. spinulosa* var. *cunninghamii* were not significantly different ($P > 0.05$) from any of the populations of *B. marginata* examined for either treatment.

There was no significant difference among any of the species for either treatment.

Water potential at turgor loss

Analysis of variance indicates a significant difference between treatments among the populations for the water potential at turgor loss. Figure 5.5 generally demonstrates that the water potential at turgor loss became less negative in the PEG stressed plants compared to the controls with the exceptions of *B. marginata* from Birches Inlet, *B. saxicola* from Wilsons Promontory and *B. spinulosa* var. *cunninghamii*.

Tukey's test for the controls indicates no significant difference ($P > 0.05$) between *B. marginata* from all of the populations and the Victorian species. In terms of the droughted seedlings, populations of *B. marginata* from Mount Wellington, Snug, Melaleuca and The Gardens and the Blowhole were significantly different from *B. spinulosa* var. *cunninghamii*.

Interpopulation physiological variation in *B. marginata* is highlighted by Tukey's test which indicates significant differences ($P < 0.05$) in the water potential at turgor loss among the control populations of *B. marginata* from Tasmania, i.e. Mount Wellington and the Gardens populations were significantly different ($P < 0.05$) from the Snug population.

In the drought treatment, *B. canei* and populations of *B. marginata* from Mount Wellington, Snug, Melaleuca, the Blow Hole and The Gardens are significantly different from *B. spinulosa* var. *cunninghamii.*

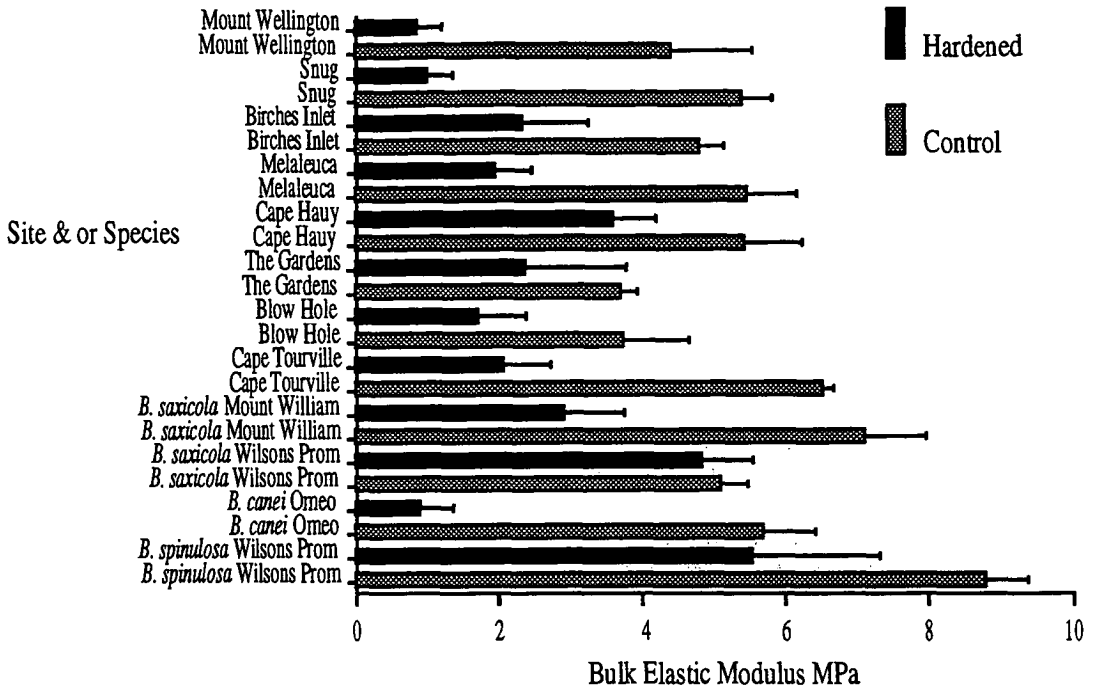

Figure 5.1. A comparison of the bulk elastic modulus *(ε)* of hardened and unhardened species of *Banksia.* Standard error bars are shown. Note that where the species is not shown it is *B. marginata.*

Table 5.2 The ratio of the adjustment in the bulk elastic modulus between unhardened and hardened specimens

Species	Unhardened : Hardened
B. spinulosa var. *cunninghamii*	~6:4
B. canei	~9:1
B. saxicola Wilsons Promontory	~5:5
B. saxicola Mount William	~7:3
B. marginata Cape Tourville	~8:2
B. marginata Blowhole	~7:3
B. marginata The Gardens	~6:4
B. marginata Cape Hauy	~6:4
B. marginata Melaleuca	~7:3
B. marginata Birches Inlet	~7:3
B. marginata Snug	~8:2
B. marginata Mount Wellington	~8:2

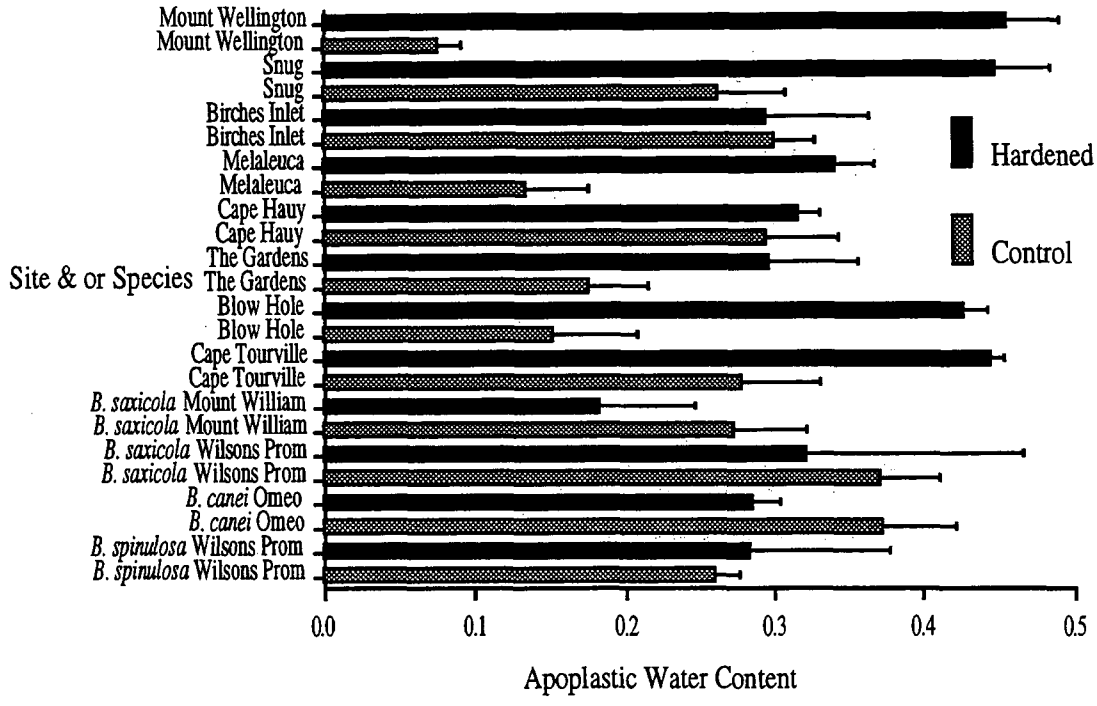

Figure 5.2. The comparison of the apoplastic water content *(R*a)* of hardened and unhardened species of *Banksia.* Standard error bars are shown. Note that where the species is not shown it is *B. marginata.*

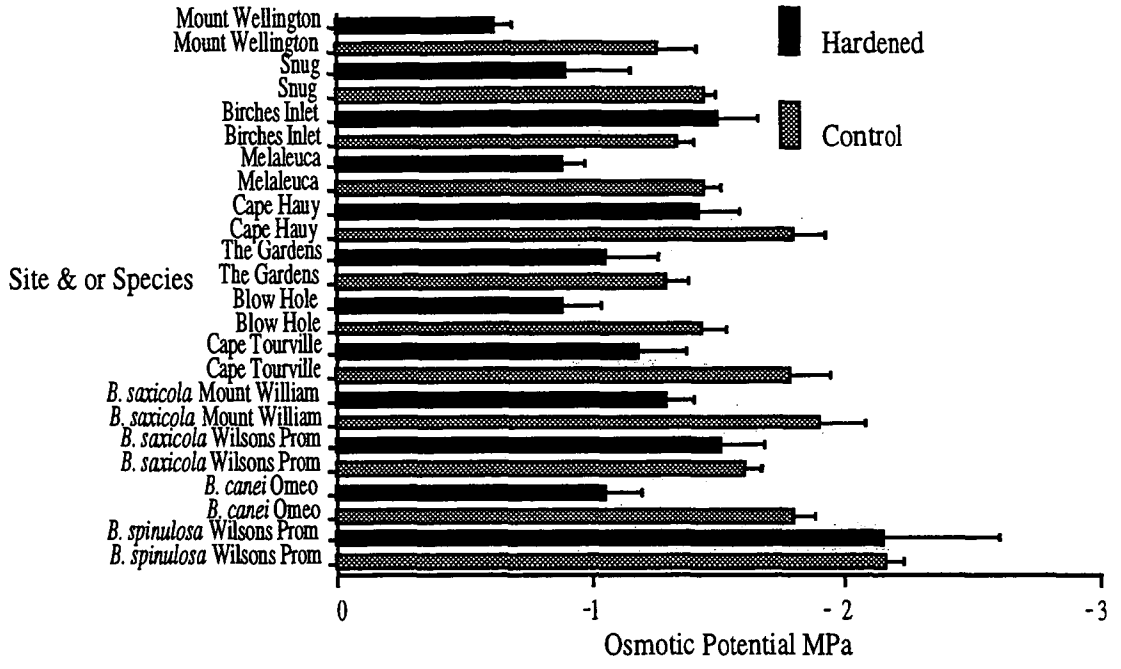

Figure 5.3. A comparison of the osmotic potential (π_O) MPa at near full turgor of hardened and unhardened species of *Banksia.* Standard error bars are shown. Note that where the species is not shown it is *B. marginata.*

Table 5.3 The ratio of the adjustment in osmotic potential between unhardened and hardened specimens

Species	Unhardened : Hardened
B. spinulosa var. *cunninghamii*	~5:5
B. canei	~6:4
B. saxicola Wilsons Promontory	~5:5
B. saxicola Mount William	~6:4
B. marginata Cape Tourville	~6:4
B. marginata Blowhole	~6:4
B. marginata The Gardens	~5.5:4.5
B. marginata Cape Hauy	~5.5:4.5
B. marginata Melaleuca	~6:4
B. marginata Birches Inlet	~4.7:5.3
B. marginata Snug	~6:4
B. marginata Mount Wellington	~7:3

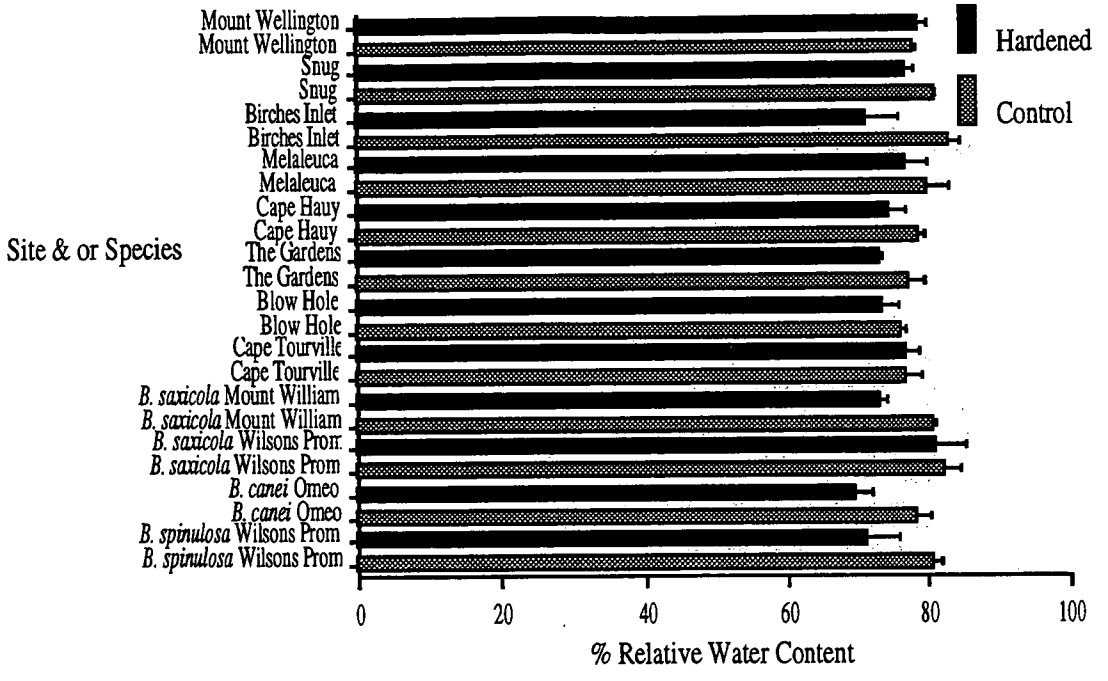

Figure 5.4. A comparison of the relative water content at turgor loss of hardened and unhardened species of *Banksia.* Standard error bars are shown. Note that where the species is not shown it is *B. marginata.*

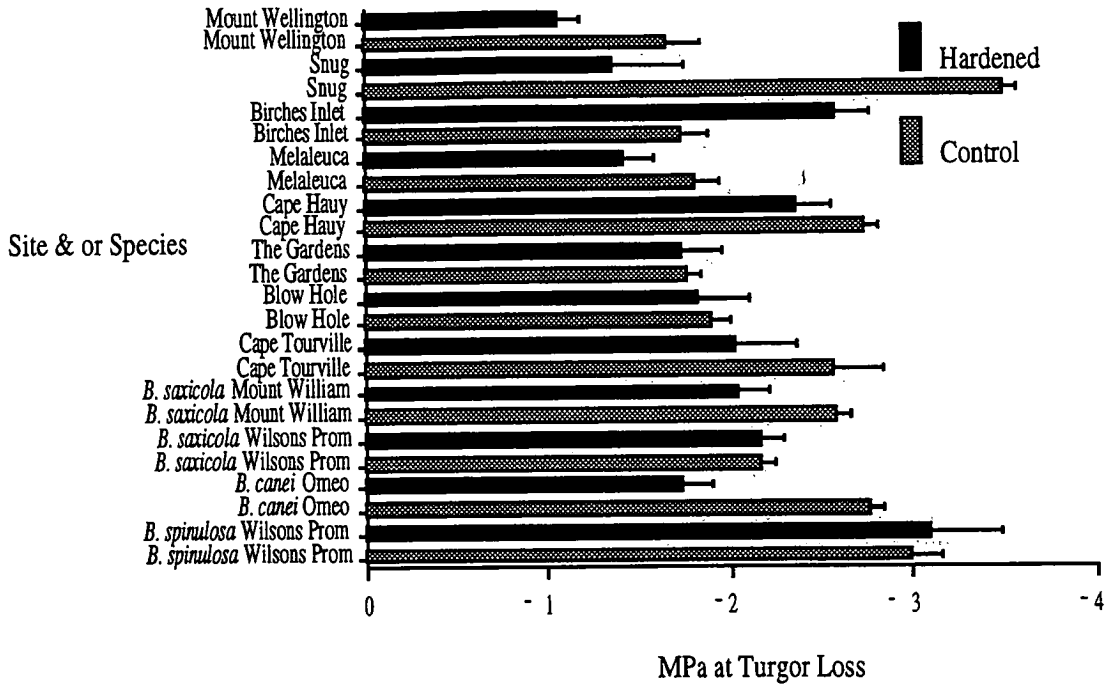

Figure 5.5. A comparison of the water potential (ψ) at turgor loss of hardened and unhardened species of *Banksia*. Standard error bars are shown. Note that where the species is not shown it is *B. marginata*.

Discussion

The distribution of terrestrial plants is more often regulated by water availability than any other environmental factor (Kozlowski 1968). This is because different species have varying levels of physiological tolerance to water stress. Consequently, the assessment and interpretation of plant water relations with respect to environmental factors can assist in the interpretation of the distributional patterns of plant species (Ritchie and Hinckley 1975).

In this study, the overall water relations results indicate little significant difference ($P > 0.05$) among the drought tolerance of the closest living relatives of the Tasmanian fossil species, (i.e. *B. spinulosa* var. *cunninghamii*, *B. canei*, *B. saxicola*) and the Tasmanian populations of *B. marginata* examined. Consequently, in view of:
(1). the current success of *B. marginata* within Tasmania,
(2). its supposed survival of the arid, glacial conditions of the Pleistocene and,
(3). the similar physiological drought tolerance of the closest living relatives of the extinct fossil *Banksia* and the Tasmanian populations of *B. marginata*, these results suggest that drought stress was unlikely to have played a major role in the Pleistocene extinction of *B. kingii* and *B. strahanensis* from Tasmania. Other water relations experiments might clarify this conclusion.

The most interesting aspect of this water relations study is that the experiments highlight two important physiological mechanisms used by the *Banksia* species examined to survive drought stress, i.e. by decreasing the value of ε and, in the case of *B. marginata* and *B. spinulosa* var. *cunninghamii*, by increasing R^*a.

Indeed, the results indicate an overwhelming trend for the closest living relatives of the fossil species and all of the PEG stressed populations of *B. marginata* to decrease ε. Figure 5.1 demonstrates that all species except *B. marginata* from The Gardens and *B. saxicola* from Wilsons Promontory underwent definite reductions in ε when drought stressed. Similarly, Andersen and McLaughlin (1991) have observed decreased ε in droughted *Picea rubens.* Andersen and McLaughlin (1991) have indicated that

decreased ε is one of the more common responses of plants to drought stress. Plants that decrease ε in response to drought stress actually improve their drought tolerance. This is because decreased ε facilitates cell turgor maintenance to lower *RWC* than otherwise (Andersen and McLaughlin 1991).

When compared to three Mediterranean sclerophyllous species, the *Banksia* species examined, whether control or drought treated, have much lower values of ε. The following are examples of the ε values of three species from Sicily at near full turgor: *Olea oleaster*, 19.51 MPa and 19.29 MPa, *Laurus nobilis*, 28.11 MPa and 40.71 MPa, and *Ceratonia siliqua*, 20.45 MPa and 24.53 MPa for the months of May and September respectively (Lo Gullo and Salleo 1988). For comparison, the highest ε value (most rigid cells) for the *Banksia* species studied was 8.8 MPa for unstressed *B. spinulosa* var. *cunninghamii* and the lowest value was 0.8 MPa for drought stressed *B. marginata* from Mount Wellington. The low ε of the *Banksia* species examined should reduce the risk of cell membrane rupture from dehydration stress.

It should be added at this point that reductions in ε result from modifications in the cell walls (Blake and Tschaplinksi 1992) in response to drought stress. Such seemingly rapid reductions in ε as witnessed in this study have also been recorded by Fan *et al.* (1994) using pressure volume curve analyses to examine pine species droughted by withholding water for eight days.

Figure 5.2 demonstrates that increases in *R*a* were mostly confined to drought stressed *B. marginata* from Tasmania with the exception of *B. spinulosa* var. *cunninghamii,* the only fossil relative which significantly increased its *R*a* in response to drought stress. An increase in the *R*a* is likely to occur in response to tissue dehydration i.e. as the apoplast dehydrates water from the symplast is transferred into the apoplast thereby causing an increase. The flow of water into the apoplasm has been reported to protect living cells from damage by protecting them from sudden water loss during subsequent dehydration (Blake and Tschaplinski 1992). As mentioned previously, high *R*a* have been observed in plants which have been pre-conditioned to water stress (Blake and Tschaplinski 1992). In addition, Vance and Zaerr (1991) have observed lower *R*a* in shaded as

Note: To accommodate the increased volume in the apoplast there must also be an increase in the size of the apoplastic compartment, i.e through anatomical change. Perhaps through cellulose fibrils loosening in some way to allow the walls to swell and contain more water (this might also be a cause for the decrease in ε values).

and Zaerr (1987) have suggested that plants with lower *R*a* have a reduced capacity to tolerate dehydration stress compared to plants with higher *R*a.* Thus, the capacity of *B. marginata* to increase its *R*a* in response to drought stress may have given this species a competitive edge over species unable to increase their *R*a* in response to the drought stress of the Pleistocene. However, as indicated previously, it is not clear whether the extinction of the fossil *Banksia* species from Tasmania during the Pleistocene was due to their sensitivity to drought.

Both decreases in ε and osmotic adjustment (i.e. the active accumulation of solutes intracellularly) have been reported to help plants maintain positive turgor *(P)* when under water stress (Hsiao 1973, Roberts *et al.* 1980, Turner and Begg 1981, Ranney *et al.* 1991, Davie *et al.* 1993, Fan *et al.* 1994). In this study however, it appears as though π_O may have a minor role in the drought tolerance of the *Banksia* species examined. For example, the π_O of all populations of *B. marginata* and all other species except *B. spinulosa* var. *cunninghamii* and *B. saxicola* from Wilsons Promontory became obviously less negative when droughted, a case of osmotic de-adjustment (Fig. 5.3). Osmotic de-adjustment has been observed in plant species sensitive to drought stress (Blake and Tschaplinski 1992). Gebre *et al.* (1994) observed less negative π_O in *Populus deltoides* after exposure to longer rather than shorter periods of wet/dry cycles. In addition, the organic solutes of this species either remained unchanged or declined after exposure to wet/dry cycles of a longer duration (Gebre *et al.* 1994).

In the case of the *Banksia* species, it is possible that the decreased ε in response to drought may compensate for the minor role that π_O appears to play in the cell turgor maintenance. Furthermore, although osmotic adjustment has been documented in many woody species (Abrams 1988, Rhizopoulous and Mitrakos 1990), its importance in terms of cell turgor maintenance has been questioned (Munns 1988). For example, Munns (1988) and Turner (1986) have both questioned the role of turgor maintenance in plant growth. According to Munns (1988) there are two lines of evidence against the role of turgor maintenance in plant growth:
(1). the turgor of tissues in growing leaves and roots in dry soils may not change even though growth is slower and,
(2). experiments which artificially manipulate turgor indicate that turgor does not control the rate of leaf expansion in saline soils. Munns (1988) maintains

these examples indicate that growth is controlled by something other than turgor.

Although Munns (1988) indicates the necessity for analysis "of the assumptions associated with the notion that osmotic adjustment is an important adaptation to dry ... conditions" it is undisputed that osmotic adjustment and decreased ε both facilitate the cell turgor maintenance of many drought stressed species. Furthermore, regardless of whether plant growth is dependent on turgor maintenance or not, turgor maintenance is vital to cell membrane stability. In particular, elastic tissues are able to shrink under drought stress, thereby maintaining *P*, which in turn helps prevent cell wall rupture and injury to the plasma membranes (Dale and Sutcliffe 1986). In this way, the physiological and biochemical processes of drought stressed plants can be safeguarded, at least in the short term. Indeed, it must also be stressed that while osmotic adjustment may assist turgor maintenance, it inhibits certain other aspects of plant performance (Roberts *et al.* 1980).

Similar to π_o, much debate has centred around the significance of ε to plant drought tolerance. For example, some authors have suggested that higher ε values rather than lower values actually assist plant survival of drought. For example, Bowman and Roberts (1985) have observed high ε values in droughted chaparral shrubs. They argue that the greater change observed in plant Ψ for a given change in tissue water volume associated with low cell wall elasticity facilitates water uptake and the prevention of cell damage from dehydration stress. However, Schulte (1992) doubts that increased ε would enhance water uptake. He maintains that water uptake in non-succulent plants is ultimately determined by the rate of transpirational loss.

Relative water contents and water potentials at turgor loss are also measures of desiccation tolerance. The more desiccation tolerant a plant, the better its chance of regaining its full metabolic functions after drought. For this experiment, the results for relative water content and water potential at turgor loss are less informative and interesting than the results for ε, R^*a and π_o. Consequently, they will be discussed briefly.

With respect to the relative water content at turgor loss, it appears as though all of the drought stressed *Banksia* species studied except *B. marginata* from Mount Wellington hardened to some extent by decreasing the relative water

content at which turgor loss occurred. The capacity to delay turgor loss to lower *RWCs* when drought stressed should enable the *Banksia* species to maintain turgor for longer periods than otherwise.

The results for the water potential at turgor loss of the droughted seedlings follow the pattern of the π_o of the droughted seedlings. This result however is of little significance to this study and will not be discussed further.

One other important aspect to discuss is the physiological variability observed among the drought tolerance of the eight different populations of Tasmanian *B. marginata*. Analysis of variance indicated significant ($P < 0.05$) physiological differences among certain of the control and drought treated populations of *B. marginata* e.g. in terms of the controls for water potential at turgor loss and *R*a* and in terms of the drought treated species for ε. However, although physiological differences were not great among the populations, the results suggest that there could be genetic differences among some of the populations examined. Indeed, genetic differences among populations of this species may account at least in part for the successful occupation of a wide range of habitats by *B. marginata* in Tasmania today (see Chapter 1, page 1, paragraph 3).

In summary the results indicate the capacity of the *Banksia* species examined to:

(1). delay turgor loss to lower *RWC* and/or,
(2). decrease ε and/or,
(3). increase *R*a*,
all of which act to improve the desiccation tolerance of *Banksia* species when droughted.

To conclude, the results highlight at least two important physiological mechanisms used by the *Banksia* species examined to deal with drought stress, i.e. decreasing ε, and more particularly, in *B. marginata* and *B. spinulosa* var. *cunninghamii* by increasing their *R*a*.

The results for this experiment do not suggest that the extinction of *B. kingii* and *B. strahanensis* from Tasmania resulted from their physiological sensitivity to the arid conditions of the Pleistocene glacial events. It is more

likely that some other biotic/abiotic factor(s) caused their extinction. However, as indicated previously, terrestrial plant distribution is more often regulated by water availability than any other environmental factor (Kozlowski 1968) and the results from the *Banksia* experiments relate to only one set of conditions. Therefore caution must be taken when interpreting the results with regard to Pleistocene *Banksia* extinctions. Furthermore, numerous other water relations experiments could have been carried out (time and practicality permitting) which may have provided a better understanding of why the fossil *Banksia* became extinct from Tasmania during the Pleistocene. For example, field water relations studies performed on adult plants would have complemented experiments on glasshouse grown seedlings, pre-dawn water potentials under drought conditions to help determine species capacity for overnight recovery of water potential, desiccation experiments could have been performed on the Victorian species, water use efficiency experiments to mention a few.

Consequently, there is still a possibility that water stress had a role in the extinction of the fossil *Banksia* species from Tasmania during the Pleistocene. This is because, as a group, vascular plants exhibit a fundamental trophic homogeneity (Knoll 1984). Consequently, when a particular resource such as water becomes limited, a plant population cannot avoid competition by choosing a new food source (Knoll 1984). Knoll (1984) thus believes competition has had a very important role in determining plant extinctions. Furthermore, purely hypothetically, and based on the more restricted distribution of the closest living relatives of the fossil species, it is possible that had the Pleistocene population size of the fossil species been smaller and more local than *B. marginata*, it is likely that climatic stress would have had a more severe impact on them than a larger, perhaps more plastic and genetically diverse species such as *B. marginata*.

Cell Membrane Stability Test

Introduction

The unusual results obtained for the π_o of the above drought stressed *Banksia* species (i.e. in most cases the π_o of the control seedlings were more negative than the droughted seedlings) prompted the testing of *Banksia* cell membrane stability *(CMS).* This was done to determine whether the PEG osmoticum used in the hydroponic medium was detrimental to the *Banksia* species. According to Blum and Ebercon (1981) and Martin *et al.* (1987) the degree of electrolyte leakage from the cells of tissues exposed to drought stress can be used as a measure of dehydration tolerance of species. It has been observed by Martin *et al.* (1987) that the tissues of dehydration tolerant plants leak fewer electrolytes in response to drought stress than less tolerant plants.

To test for cell membrane stability, the PEG test of Premachandra *et al.* (1993) was followed. They used this test to assess drought tolerance in orchard grass. The PEG test has also been used to assess plant drought tolerance by Blum and Ebercon (1981), and Premachandra and Shimada (1987).

The aim of this experiment was to determine whether the comparatively less negative π_o of the drought stressed seedlings could have resulted from the leakage of electrolytes from damaged root cells into the hydroponic medium. Leakage of electrolytes into the hydroponic medium may have resulted if PEG induced cell membrane damage had occurred in the *Banksia* seedlings.

The PEG test, similar to the frost and desiccation methods described in Chapter 4, is another modification of the electrical conductivity method used by Dexter *et al.* (1930).

Method

Six plants per species and/or population of *B. marginata* were randomly selected from the glasshouse grown specimens. Five, 6 mm discs were removed from the leaf tissue of each seedling to form one unit. The discs were washed thoroughly in de-ionised water and each disc unit was submerged for 24 hours in glass vials containing 10 ml of -4.8 and -6.0 MPa of PEG 3350 solution (see Mexal *et al.*1975 for %W/V of PEG). After incubation, the leaf discs were washed four times with de-ionised water and submerged in clean glass vials with 10 ml of de-ionised water which were then shaken at room temperature for 24 hours. After 24 hours the conductivity of the solution in the vials was measured. Discs in solution were then microwaved to ensure maximum tissue damage and were shaken for another 24 hours. At the end of this time, the conductivity was re-measured. Please refer to the frost and desiccation sections of Chapter 4 for a more detailed description of the technique and the methods used for data analysis.

Statistics

A one way Anova using SAS (SAS Institute Inc. 1988) was performed to determine whether there was any significant difference between the electrolyte leakage from the controls and the PEG treated discs.

Results and Discussion

The results indicated that no obvious ($P > 0.05$) leakage of electrolytes occurred for any of the *Banksia* species examined at levels of drought stress far exceeding those used in the hydroponic water relations experiments of Chapter 4 i.e. -1.0 MPa compared to -4.8 and -6.0 MPa. This result therefore suggests that the PEG 3350 concentrations used in the previous and following chapters were probably not harmful to the seedlings examined.

CHAPTER 6. FROST TOLERANCE EXPERIMENTS

Introduction

Frost and drought are important forcing features in natural selection. For reproductively viable populations of *Banksia kingii* and *B. strahanensis* to have survived the glacial events of the Pleistocene the capacity to harden and tolerate frost would have been essential.

There are many ways plant species survive cold stress. The two most common strategies are the active accumulation of solutes and the transference of water from the symplast to the apoplast. The active accumulation of solutes intracellularly is similar to the process of osmotic adjustment which occurs in some drought hardened plants. Plants which respond to freezing stress in this way have been termed "freezing sensitive" Larcher (1980). The active accumulation of solutes enables these plants to tolerate lower temperatures than they would otherwise, before the onset of intracellular freezing. By contrast, plants that avoid intracellular ice crystal formation by the displacement of symplastic water to the extracellular environment are termed freezing tolerant. Freezing tolerant plants also tend to be dehydration tolerant.

Occasionally, when frosts are particularly severe, the mechanisms employed by many plant species to deal with cold stress are inadequate and tissue damage from intracellular ice crystal formation may occur. The growth of ice crystals intracellularly can result in cell wall rupture, the leakage of symplastic fluid from the cells (Vasil'yev 1961, Larcher 1980), and in some cases irreparable tissue damage results in plant death. It can be expected that during glacials, the population numbers of many species will decline, with the natural selection of frost tolerant individuals over individuals less frost tolerant. Some frost tolerant species may even expand their range during glacials, other species will survive in refugia and many frost sensitive species will decline in numbers to the point of extinction. The capacity of species to survive climatically stressful periods such as glacials, will depend on the general physiological hardiness of the species, their competitive abilities, the size of the original population, the gene pool diversity and to some extent on chance.

The aim of this study was to determine whether the extinction of *B. kingii* and *B. strahanensis* from Tasmania during the Pleistocene was likely to have been a consequence of their physiological sensitivity to cold stress. As in the water relations study, the cold tolerance of the currently successful *B. marginata* was used as a benchmark to determine how frost sensitive the closest living relatives of the fossil species may have been.

Frost Experiments

Five plants, 15-18 months old, were randomly selected from each of the same batches as previously grown for the experiments of Chapter 5.

The selected plants were grown hydroponically in 10 L of aerated half strength Hoagland's solution for three weeks prior to experimentation as detailed in Chapter 4. Frost experiments (1-4) were performed in a hydroponic system so the results could be compared with the experiments requiring PEG 3350 treatments.

Apical shoots were removed from the *Banksia* seedlings and labelled on the day of the experiments and placed in containers of de-ionised water. The foliage used was that which had been produced during the previous growing season. Particular care was taken with those specimens grown in PEG 3350 to remove any PEG residue which had splashed onto the leaf surfaces. All the leaves were washed twice in de-ionised water and blotted dry before use. Refer to Chapter 4 for details of the frost method, equipment, data and statistical analysis used.

For the hydroponic frost experiments described in this chapter, five leaf discs (each 6 mm diameter) were taken per plant using a hole puncher (avoiding the midrib in all cases except *B. spinulosa* var. *cunninghamii* and *B. marginata* from Melaleuca and Birches Inlet which have leaves which are too narrow). The five discs together represented one sample. Each sample from each species from each population was artificially frosted in the frost cabinet described in Chapter 4 at -4, -7, -9, -12, -15°C. Methods from this stage follow those described in Chapter 4. The difference in the number of leaf discs used per shoot between the frost and desiccation experiments

relate to the fact that there was not enough space to put any more than two discs per sample in the desiccators.

A control was set up in which specimens were prepared exactly as above but were not frost treated.

Experiment 1

Plants were cold hardened only. They were grown hydroponically in a controlled environment with a 12 hour photo-period, at a constant day/night temperature of 5°C and 2°C respectively, at a light intensity of 300 μ Einsteins m^{-2} s^{-1}. Trunova (1982) indicates that plants usually start to harden at temperatures slightly above 0°C, i.e. around 2°C, after being exposed for a minimum of one to two weeks.

The aim of this experiment was to compare the frost tolerance of each species under controlled conditions to get an indication of their potential field survival during the glacial events of the Pleistocene.

Experiment 2

Plants were drought stressed but not cold hardened. They were grown hydroponically in a controlled environment for total of three weeks under a 12 hour photo-period with a constant day/night temperature of 12°C and 10°C respectively at a light intensity of 300 μ Einsteins m^{-2} s^{-1}. The plants were droughted by the same procedure described in Chapter 5.

The aim of this experiment was to compare the frost tolerance of the different *Banksia* species under drought stress. As indicated previously, drought would have been a common form of stress affecting the growth of these species during the glacial events of the Pleistocene. Furthermore, Vasil'yev (1961) has indicated that temporary drought will often increase the frost tolerance of plants.

Experiment 3

Plants were both cold hardened and exposed to drought stress. They were grown hydroponically for three weeks, under a 12 hour photo-period, at a constant day/night temperature of 5°C and 2°C respectively, at a light intensity of 300 μ Einsteins m^{-2} s^{-1}. The plants were droughted by the same procedure described in Chapter 5.

The aim of this experiment was to compare the frost tolerance of *Banksia* spp. which had been cold hardened and drought stressed simultaneously to see if both cold hardening and drought stressing produced a synergism i.e. resulting in an even greater frost tolerance than if given either drought or cold as a single treatment only.

Experiment 4

Plants were neither drought stressed nor hardened to cold. They were grown hydroponically in a growth cabinet for three weeks with a 12 hour photo-period at constant day/night temperature of 12°C and 10°C respectively, at a light intensity of 300 μ Einsteins m^{-2} s^{-1}.

The aim of this experiment was to try to determine how the different Pleistocene *Banksia* spp. may have coped with out of season frosts during the glacial and interglacial events of that period. Sudden freezes and out of season frosts have been responsible for much of the frost damage which occurs in southern France, Portugal and south-east United States. Raymond *et al.* (1992) suggest that certain species of plants should be evaluated for their frost tolerance in the unhardened state, particularly crop plants.

Experiment 5

For this experiment, seedlings within pots were placed at an altitude of 600 m asl for a two month period. The plants were watered twice weekly to supplement natural precipitation and were sampled to determine their frost tolerance after four and eight weeks of hardening under "natural" conditions.

The plants were randomly placed within a wire exclosure on the eastern side of Mount Wellington. The plants would have been exposed to the full force of

the weather over the winter months of July and August. Monthly maximum and minimum temperatures were recorded to give an indication of the temperature extremes the plants were exposed to (see Table 6.2.).

Table 6.2 Weekly Maximum and minimum temperatures at 600 m asl for July and August 1994.

Month	July	July	July	July	August	August	August	August
Max	9°C	8°C	10°C	11°C	10°C	10°C	11°C	11°C
Min	-3°C	-2°C	-4°C	-4°C	-5°C	-3°C	-2°C	-2°C

The aim of this experiment was to determine whether the frost tolerances of the artificially hardened specimens were comparable to the frost tolerances of specimens in the winter hardened specimens at 600 m asl.

Data Analysis

Probit Transformation

A probit transformation was performed on the *It* data. From the probit transformed frost *It* data it was possible to estimate the *T50* values (i.e. the temperature at which each plant examined succumbed to 50% tissue damage).

Statistical Analysis

A one way ANOVA with *a posteriori* comparisons was performed on the *T50* values with Tukey's method using SAS (SAS Institute Inc. 1988) to determine whether there were any significant differences among the frost tolerance of the seedlings under examination.

Results

Artificial cold hardening treatment

Figure 6.1 shows that the artificially cold hardened seedlings of the Victorian species, *B. spinulosa* var. *cunninghamii*, *B. canei* and *B. saxicola* from

Wilsons Promontory, were less frost tolerant (i.e. less negative *T50* values) than the seedlings of *B. marginata* from the eight Tasmanian populations. Tukey's test (see Appendix 2) indicates that *B. spinulosa* var. *cunninghamii, B. canei* and *B. saxicola* from Wilsons Promontory were significantly less ($P < 0.05$) frost tolerant than *B. marginata* from Birches Inlet, Melaleuca, Mount Wellington, Snug, and The Gardens. In addition, *B. spinulosa* var. *cunninghamii, B. canei* and *B. saxicola* from Wilsons Promontory were significantly different ($P < 0.05$) from the Blow Hole population.

For comparison, Figure 6.1 demonstrates that seedlings of *B. spinulosa* var. *cunninghamii* were the least frost tolerant (*T50* of -7.50°C) while *B. marginata* from the coastal site of Cape Tourville had the greatest frost tolerance (*T50* of -11.44 °C).

Tukey's test (see Appendix 2) indicates significant differences ($P < 0.05$) in the frost tolerance of the different populations of Tasmanian *B. marginata* seedlings for this treatment, i.e. *B. marginata* from Cape Hauy was significantly different ($P < 0.05$) from *B. marginata* from Birches Inlet, Melaleuca, Mount Wellington, Snug and The Gardens.

Artificial drought stress treatment

Figure 6.2 also shows that the Victorian seedlings of *B. spinulosa* var. *cunninghamii* and *B. saxicola* from Wilsons Promontory were the least frost tolerant. However, the other Victorian seedlings, i.e. Mount William *B. saxicola* and *B. canei* were the most frost tolerant for this treatment, e.g. a *T50* of -25.45°C and -18.25°C respectively.

For this treatment, Tukey's test indicates that *B. canei* and *B. saxicola* from Mount William were significantly more frost tolerant ($P < 0.05$) than *B. spinulosa* var. *cunninghamii* and *B. saxicola* from Wilsons Promontory. In addition, *B. saxicola* from Mount William was significantly ($P < 0.05$) more frost tolerant than all populations of *B. marginata.* There was no significant difference ($P > 0.05$) in frost tolerance among the populations of *B. marginata.*

The frost tolerance of all of the species and populations of *B. marginata,* except the seedlings from the Cape Tourville population, were greater after

the drought stress treatment than the cold hardening treatment (Fig. 6.1 and Fig. 6.2). The noticeable differences in the response of the seedlings to the drought compared to their response to the cold treatments is not easy to explain, especially since the physiological response to drought and cold stress is often similar.

Artificial cold hardening and drought stress treatment

Tukey's test indicates that the frost tolerance of *B. saxicola* from Mount William is significantly less ($P < 0.05$) than *B. marginata* from Cape Hauy and Snug. Furthermore, the results for this treatment indicate no significant difference ($P > 0.05$) in frost tolerance among the different populations of *B. marginata* from Tasmania.

No obvious treatment synergism occurred for this treatment i.e. the combined treatments did not confer on the *Banksia* populations a remarkedly greater level of frost tolerance then either of the drought or cold hardening treatments on their own, e.g. the seedling frost tolerance of the Mount William *B. saxicola* population was greater for drought alone at -25.24°C compared to -8.63°C for the drought/cold hardened treatment. In fact, the frost tolerance of all of the populations of *Banksia* were greater after the drought treatment alone when compared to the drought/cold hardening treatment with the exception of *B. spinulosa* var. *cunninghamii* and *B. saxicola* from Wilsons Promontory and *B. marginata* from Cape Hauy and Cape Tourville, see Figs 6.2 and 6.3. The frost tolerance of all the other Victorian species and populations of *B. marginata* (except from Melaleuca, Mount Wellington and The Gardens) however, were greater after the simultaneous treatment than after cold hardening alone (Figs 6.1 and 6.3). This implies that the effect of the drought stress treatment on the *Banksia* species examined was perhaps more effective in improving the frost tolerance of the *Banksia* then the cold hardening treatment on its own.

Out of season frost treatment

Figure 6.4 indicates that the seedlings of *B. saxicola* from Mount William and Wilsons Promontory were the least frost tolerant for this experiment. In fact, the frost tolerance of *B. saxicola* from these two populations was

considerably poorer for this than for all of the other experiments e.g. the *T50* for the Wilsons Promontory population ranged from -8.22°C (cold hardened only), -9.73°C (drought stressed only), and -10.09°C (cold hardened and drought stressed) compared to -7.8°C for the out of season frost treatment. *Banksia saxicola* from Mount William was significantly less ($P < 0.05$) frost tolerant than *B. marginata* from Cape Tourville, Cape Hauy, the Blowhole, Mount Wellington, Melaleuca, The Gardens, Snug and Birches Inlet, *B. canei* and *B. spinulosa* var. *cunninghamii*. *Banksia saxicola* from Wilsons Promontory was significantly less ($P < 0.05$) frost tolerant than *B. spinulosa* var. *cunninghami*, *B. marginata* from Mount Wellington, Melaleuca, The Gardens, Snug and Birches Inlet.

Banksia marginata from Birches Inlet was significantly ($P < 0.05$) more frost tolerant than *B. marginata* from Cape Tourville, Cape Hauy and the Blow Hole. The Snug and The Gardens populations were significantly different ($P < 0.05$) from the Cape Tourville and Cape Hauy populations. The Melaleuca population was significantly different ($P < 0.05$) from the Cape Tourville population.

Natural cold hardened plants at 600 m altitude for four weeks.

Figure 6.5 indicates that *B. spinulosa* var. *cunninghamii* was the least frost tolerant species after four weeks' winter hardening on Mount Wellington. Indeed, *B. spinulosa* var. *cunninghamii* was significantly less ($P < 0.05$) (see Tukey's test in Appendix 2) frost tolerant than seedlings of *B. canei*, *B. saxicola* from Mount William and Wilsons Promontory and *B. marginata* from Cape Hauy and Cape Tourville, The Gardens, Melaleuca and Snug.

There was no significant ($P > 0.05$) variation among the populations of *B. marginata* from Tasmania for this treatment. The frost tolerance of *B. canei* and *B. saxicola* from Mount William and Wilsons Promontory were not significantly different ($P > 0.05$) from *B. marginata* from the Blowhole, Mount Wellington, Cape Hauy, Birches Inlet, The Gardens, Melaleuca and Snug. The frost tolerance of the "mountain hardened seedlings" was greater overall than the frost tolerance of the artificially cold hardened only seedlings.

Natural cold hardened for eight weeks at 600 m asl.

Figure 6.6 indicates that *B. spinulosa* var. *cunninghamii* was the second least frost tolerant of the species examined for this experiment. *Banksia marginata* from the Blowhole was the least frost tolerant with a *T50* of -9.14°C. Tukey's test (see Appendix 2) indicates *B. spinulosa* var. *cunninghamii* was not significantly ($P > 0.05$) less frost tolerant than all the other species and populations of *B. marginata* examined. Tukey's test indicates less variation in the frost tolerance among the different species and populations of *B. marginata* examined after eight weeks of natural hardening than four weeks. The frost tolerance of the different species and populations of *B. marginata* did not necessarily improve with the duration of hardening since some populations had better frost tolerance after eight weeks while others had better frost tolerance after four weeks.

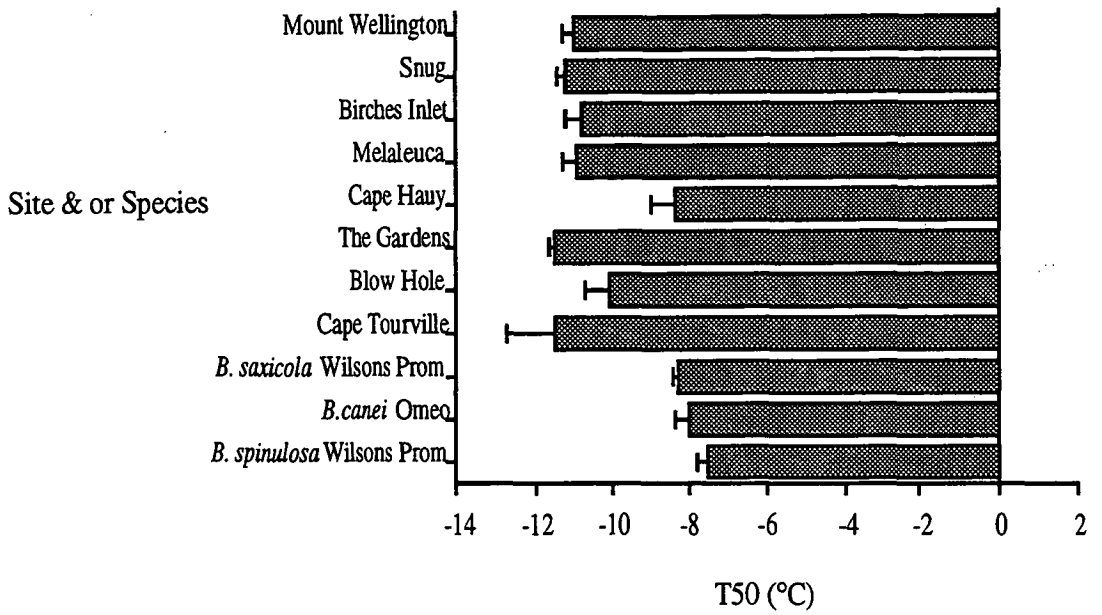

Figure 6.1. A comparison of the frost tolerance (°C at 50% tissue damage) of cold hardened species of *Banksia.* Standard error bars are shown. Note that where the species is not shown it is *B. marginata.*

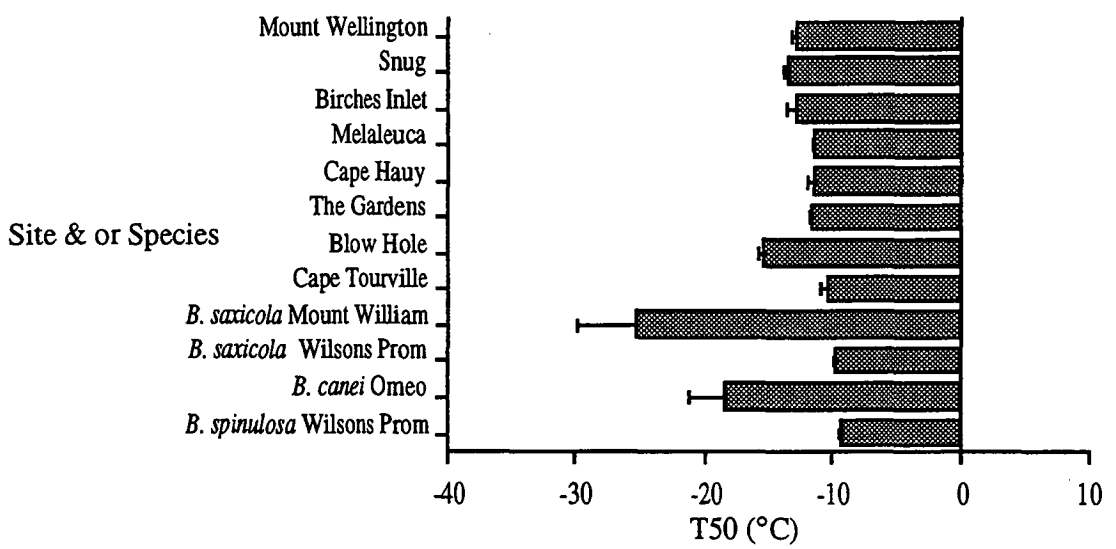

Figure 6.2. A comparison of the frost tolerance (°C at 50% tissue damage) of drought hardened species of *Banksia.* Standard error bars are shown. Note that where the species is not shown it is *B. marginata.*

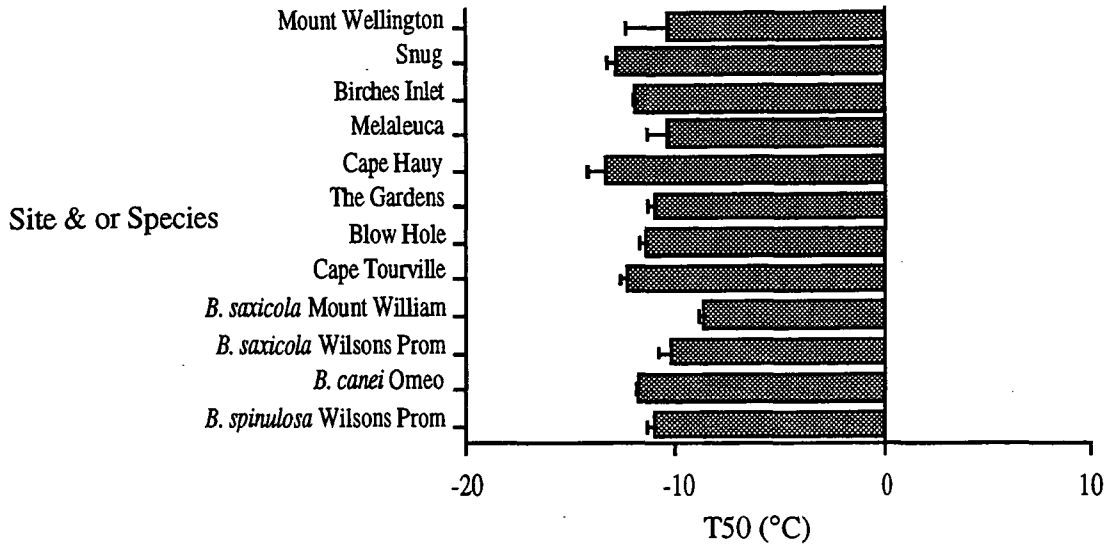

Figure 6.3. A comparison of the frost tolerance (°C at 50% tissue damage) of cold hardened and drought stressed species of *Banksia*. Standard error bars are shown. Note that where the species is not shown it is *B. marginata*.

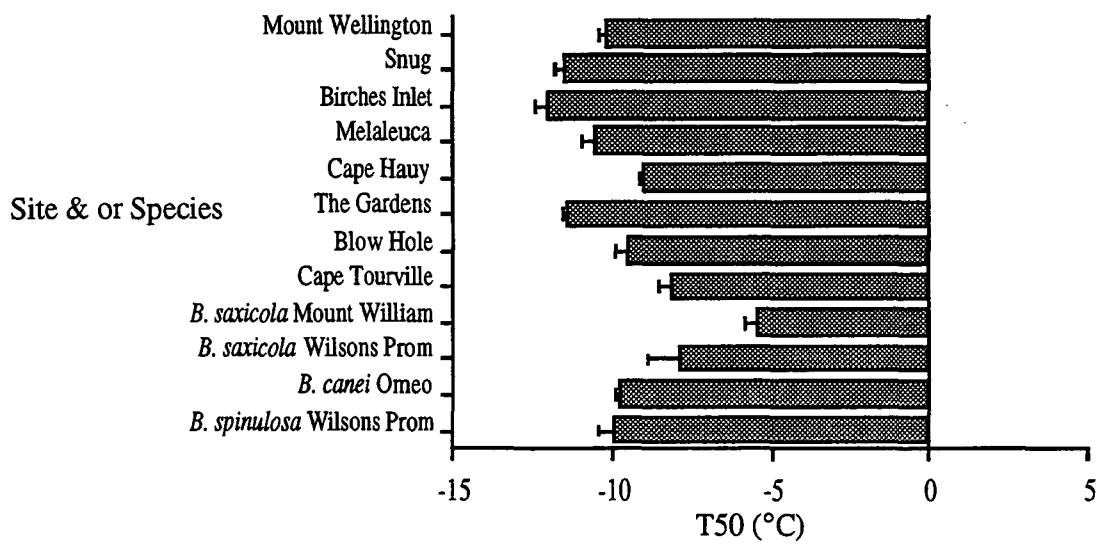

Figure 6.4. A comparison of the frost tolerance (°C at 50% tissue damage) of unhardened species of *Banksia*. Standard error bars are shown. Note that where the species is not shown it is *B. marginata*.

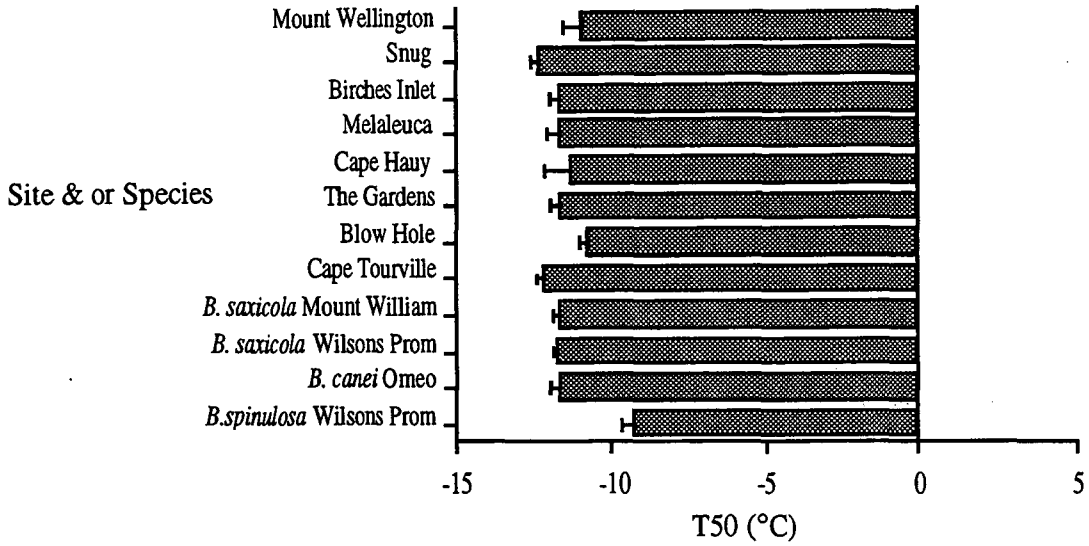

Figure 6.5. A comparison of the frost tolerance (°C at 50% tissue damage) of *Banksia* species after four weeks of winter hardening at 600 m asl. Standard error bars are shown. Note that where the species is not shown it is *B. marginata.*

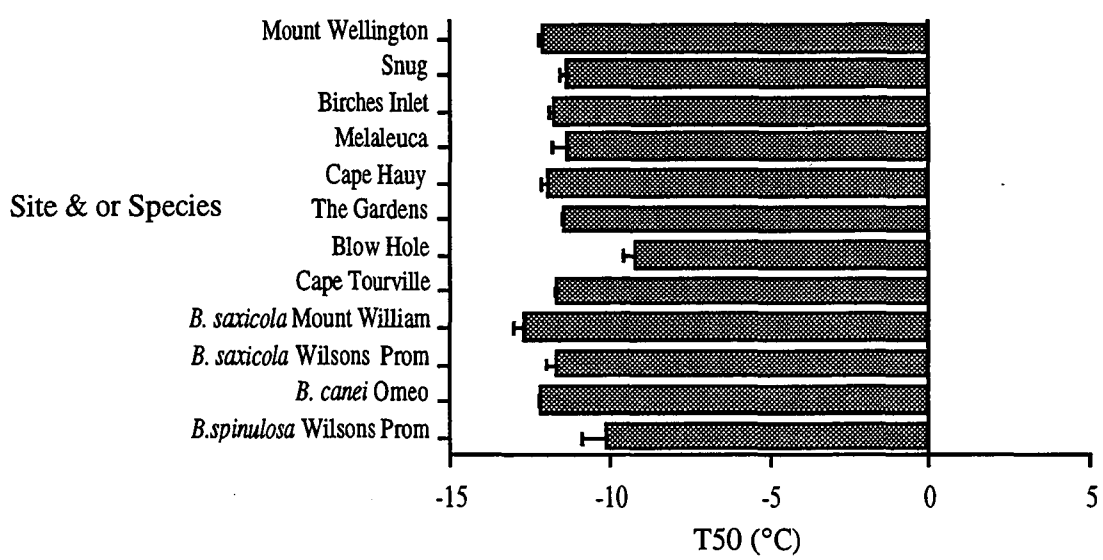

Figure 6.6. A comparison of the frost tolerance (°C 50% tissue damage) of *Banksia* species after eight weeks of winter hardening at 600 m asl. Standard error bars are shown. Note that where the species is not shown it is *B. marginata.*

Discussion

The lowest freezing temperature a plant can survive is thought to be related to the ability of its tissues to endure the dehydration stress which accompanies the withdrawal of water from the cells into the apoplast (Marentes *et al.* 1993). Indeed, frost tolerant plants when subject to cold stress have been reported to respond in a similar way to drought tolerant plants (Sakai and Larcher 1987). Some of these responses include modifications to the plasmalemma (Sakai and Larcher 1987) and osmotic adjustment (Levitt 1972, Sakai and Larcher 1987). More recently, several lines of evidence suggest that apoplastic proteins also have an important role in defining the limits of a species' frost tolerance (Marentes *et al.* 1993).

The results for the frost experiments are only slightly more conclusive than the water relations experiments about the possible fates of *B. kingii* and *B. strahanensis* during the Pleistocene.

The frost results are complex. It appears as though the extinction of both these fossil species may have been caused by different types of climatic stress. For example, the results indicate that seedlings of *B. saxicola* (closely related to *B. kingii*) from Mount William and Wilsons Promontory were particularly sensitive to the out of season frost treatment. Out of season frosts may have occurred at any time during the Pleistocene, i.e. during both the glacial and interglacial periods. Indeed, there is a lot of evidence to suggest that out of season frosts play a major role in plant distribution patterns. For example, growing season frosts have been recognised as potentially important determinants of the distribution of certain eucalypt species (Costin 1954, Burke *et al.* 1976). Additionally, Christersson *et al.* (1987) maintain that while frost injury to forest trees in Sweden does not occur during the winter, forest trees in Sweden are susceptible to damage from late spring, summer and early autumn frosts.

The different populations of unhardened Tasmanian *B. marginata* have comparatively better frost tolerance than *B. saxicola* for all treatments except where specimens were hardened at 600 m asl and for the drought stress only treatment where *B. saxicola* from Mount William is the most frost tolerant. *Banksia spinulosa* var. *cunninghamii* (closely related to fossil *B.*

strahanensis) appears to be the least frost tolerant of the species examined overall. For comparison, after three weeks exposure to artificial cold this species was the least frost tolerant. Additionally, for this treatment the Victorian species *B. canei* and *B. saxicola* from Wilsons Promontory were the two next least frost tolerant species. Indeed, the overall, frost tolerance of the artificially cold hardened populations of *B. marginata* were significantly greater ($P<0.05$) than the frost tolerance of the closest living relatives of *B. kingii* and *B. strahanensis*. The apparent inherent frost tolerance of *B. marginata* should enable it to cope with out of season frosts under natural conditions.

In addition, artificially drought stressed seedlings of *B. spinulosa* var. *cunninghamii* were less frost tolerant than all of the other *Banksia* species e.g. with a *T50* of -9.09°C compared to -25.45°C for *B. saxicola* from Mount William (see Fig. 6.2). However, the frost tolerance of drought stressed *B. spinulosa* var. *cunninghamii* was not significantly different ($P>0.05$) from any of the populations of Tasmanian *B. marginata* or any of the other species, except *B. saxicola* from Mount William and *B. canei*.

The frost tolerance of *B. spinulosa* var. *cunninghamii* after four and eight weeks of cold hardening at 600 m asl indicated a trend for this to be the least or near least frost tolerant species. For example, after four and eight weeks hardening, *B. spinulosa* var. *cunninghamii* was the least and second least frost tolerant of the species respectively (see Figs 6.5 and 6.6). *Banksia spinulosa* var. *cunninghamii*, was significantly less ($P < 0.05$) frost tolerant after four weeks than seedlings from the following *Banksia* populations: *B. canei*, *B. saxicola* from Mount William and Wilsons Promontory, *B. marginata* from Cape Hauy, Birches Inlet The Gardens, Melaleuca, Cape Tourville and Snug.

After eight weeks of hardening, *B. spinulosa* var. *cunninghami* was not significantly different ($P < 0.05$) from the other species and populations of *B. marginata*.

At this stage, it should be stressed that according to the fossil record, *B. strahanensis* (the fossil relative of *B. spinulosa* var. *cunninghamii*) is thought to have become extinct from Tasmania during the early Pleistocene. According to Fitzsimons and Colhoun (1991), the most extensive Pleistocene

glaciation occurred in the early or middle Pleistocene. Therefore, these results suggest that the earlier Pleistocene extinction of *B. strahanensis* from Tasmania than *B. kingii* may be a function of a greater sensitivity to frost than the other species, however, these results are not conclusive.

It is possible that the poorer frost tolerance generally exhibited by *B. spinulosa* var. *cunninghamii* is an artefact of the leaf disc preparation for the frost procedure. By the nature of its narrow leaves, the midrib of *B. spinulosa* var. *cunninghamii*, and *B. marginata* from Melaleuca and Birches Inlet were unavoidably incorporated into the discs. Had the midrib not been incorporated into their leaf discs these species may have exhibited greater frost tolerance i.e. because there would have been less electrolyte leakage from the xylem and phloem of the midrib into the vials. It should be pointed out however, that the frost damage of *B. marginata* from Melaleuca and Birches Inlet was not especially different from the other populations of *B. marginata*. Furthermore, the control leaf discs also included the midrib and the *It* of the frosted discs were weighted by the control.

The poorer frost tolerance displayed by *B. spinulosa* var. *cunninghamii* could have an anatomic basis, i.e. this species has less supportive structure in its leaves than the other species examined (G.J. Jordan pers. comm.) perhaps making its cells more susceptible to damage from ice crystal formation.

With respect to the out of season frost however, *B. spinulosa* var. *cunninghamii* appears to be relatively tolerant with a *T50* of -9.96°C. The *T50* of *B. spinulosa* var. *cunninghamii* generally does not change much from treatment to treatment, except that the *T50* of the artificially cold hardened seedlings was quite different (-7.49°C) from the other treatments: e.g. -11.29°C (simultaneous exposure to cold and drought stress), -9.17°C (four weeks at 600 m asl), and -10.11°C (eight weeks at 600 m asl). It is unusual that the artificially cold hardened seedlings of *B. spinulosa* var. *cunninghamii* should be less frost tolerant than the seedlings subject to the out of season frost treatment.

Banksia saxicola from Mount William and *B. canei* have their best frost tolerance when exposed to moderate drought stress by the PEG treatment. This result is supported by Vasil'yev (1961) who has observed improved frost tolerance in certain plants exposed to drought stress. Nelson *et al.* (1993)

have also observed that when water is withheld from *Simmondsia chinensis* in autumn, winter flower bud injury is reduced and that seed and seed wax yields are improved following cold winters. Similarly, by imposing water stress on lemons, limes and guavas for two to three weeks in autumn their cold hardiness was increased (Yamada 1989). In addition, as will become apparent in the following chapter, the frost tolerance of *B. marginata* when examined over a 14 month period, was actually greatest for the summer month of February, hence also hinting at the capacity for improved frost tolerance with drought in *B. marginata.*

From these frost results, it appears that the seedlings of *B. saxicola* from Mount William and *B. canei* are less likely to be injured by frost while under some degree of drought stress. As has already been indicated, the climate of Tasmania during the glacial events of the Pleistocene was much drier than at present, particularly toward the east coast of Tasmania. It may be that the regular exposure of *B. kingii* to drought stress during the glacials of the Pleistocene would have improved its chances of surviving out of season frosts, thereby enabling it to survive later into the Pleistocene than the apparently less frost tolerant *B. strahanensis.* It should be pointed out however, that the fossil sites where both *B. kingii* and *B. strahanensis* are reported to have occurred currently have very high rainfalls. By contrast, known fossil sites for the east coast of Tasmania are very rare, but several refugia for plant species thought to have been more widespread during the dry Last Glacial have been identified by Kirkpatrick and Brown (1984). Perhaps *B. kingii* was unsuccessful in retracting to these dry refugia during the Pleistocene.

To conclude, this study only hints at the possible causes of the Pleistocene extinction of *B. strahanensis* and *B. kingii* from Tasmania.
The results however, are not conclusive, although it does appear from the combination of experiments performed that:
(1). B. spinulosa var. *cunninghamii* (closely related to *B. strahanensis*) is overall the least frost tolerant species and,
(2). B. saxicola (closely related to *B. kingii*) is the species most susceptible to out of season frost and,
(3). the frost tolerance of *B. saxicola* from Mount William and *B. canei* is more substantially improved by drought stress than the frost tolerance of the eight benchmark populations of Tasmanian *B. marginata.*

It is possible that the results obtained for the *Banksia* spp. merely reflect genetic adaptation of their physiological characteristics to suit their current habitats. For example, it could be argued that the comparatively poorer frost resistance observed in *B. spinulosa* var. *cunninghamii* (population from 200 m asl) is a function of the inherent adaptation of the seedlings to the coastal habitat of their parent plants, i.e. in general, coastal climates of a certain land mass are less cold than inland climates. Theoretically, coastal populations of plants should be less frost tolerant than inland populations of the same species. However, *B. saxicola* from Wilsons Promontory (250 m asl) and Mount William (1180 m asl) don't always follow this pattern. For example, there were certain treatments where the Mount William population was less frost tolerant than the coastal population, e.g. for the simultaneous cold hardening/PEG (not significantly differant) and out of season frost treatments (significantly different) (Figs 6.3 and 6.4). Furthermore, it is also apparent that not all plant species are physiologically adapted to their current day habitats and in some cases, the ecophysiology of a species more accurately reflect an adaptation to past rather than present climatic conditions (Read and Hill 1988, Blake and Jordan 1993).

The frost results, similar to the water relations results, highlight significant differences ($P < 0.05$) among the populations of *B. marginata* for certain treatments, but the population differences are of a limited magnitude. In the next chapter, more obvious significant physiological differences are reported among field populations of mature *B. marginata* along an altitudinal cline. Thus, although the water relations and frost experiments do not provide conclusive evidence that the extinction of *B. kingii* and *B. strahanensis* from Tasmania during the Pleistocene was the result of their physiological weakness to drought and/or cold stress, the results begin to expose the potential physiological competitiveness of *B. marginata*. This potential is demonstrated by its inherent frost and drought hardiness and most importantly by the obvious population differences in *B. marginata* for some of the treatments. These differences perhaps reflect the genetic diversity of this species and may thus account for its successful occupation of a wide range of habitats today.

The artifical hardening of the *Banksia* to drought and cold was useful in comparing the frost tolerance of the different species and populations of *B.*

marginata. However, total confidence should not be placed in the actual *T50* values obtained, rather, more significance should be placed on the trends observed in the frost tolerance of the species.

There will always be difficulties regarding the level of confidence that should be placed in results obtained from controlled environment experiments. This uncertainty however, is equally applicable to results obtained from field studies because the interaction between plants and their physical environment is so complex. This complexity is evident even in the simple controlled study discussed here i.e. where plant response to two physiological factors was examined. For example, where the *Banksia* species were hardened to cold and exposed to drought simultaneously, it was expected that the combined treatments would result in *Banksia* with superior frost tolerance compared to *Banksia* treated for just one of the treatments. This however, was not generally the case, (see Figs 6.1-6.5). Only *B. spinulosa* var. *cunninghamii* and *B. saxicola* from Wilsons Promontory and *B. marginata* from Capes Hauy and Tourville displayed greater frost tolerance in response to the combined treatments compared to either the drought or cold treatment on their own.

The results obtained from the other experiments, i.e. artifical cold hardening, "natural" cold hardening, artifical drought stress and out of season frost were more sensible in terms of plant response. Firstly, the *T50* values for artifically cold hardened *Banksia* were not too different from the values obtained after four and six weeks "natural hardening" at 600 m asl: For example, *T50* values ranged between -7 to -12°C, -9 to -13° and -9 to -13.5°C respectively. Secondly, based on the observations that physiological plant response to drought and cold can be similar, it was expected that the exposure of the *Banksia* species to drought would have improved the frost tolerance of at least some of the species and populations of *B. marginata* examined, which it did, with the response of *B. saxicola* from Mount William and *B. canei* being of particular note. Finally, where the plants were subjected to out of season frosts, it was reasonable to expect that at least some of the species would have demonstrated lower levels of frost tolerance than if they had they been pre-hardened. In keeping with this expectation, for example, the frost tolerance of *B. saxicola* from Mount William was much lower for this experiment than for all of the others, see Figs 6.1-6.5.

CHAPTER 7 THE CLINE

Introduction

Most plant species occupy sites where environmental conditions change from less to more stressful throughout the year (Mooney 1980). Ecophysiological studies of the response of plants to seasonal fluctuations in their natural habitats can provide important clues to further understanding the mechanisms used by different plant species to survive stressful periods (Mooney 1980). Mooney (1980) maintains that it is an evolutionary fact that no single genotype can operate efficiently over a wide range of environmental conditions. However, he concedes some species are more generalist or plastic than others and are able to acclimate to a wide range of habitat conditions.

An ecophysiological study of the response of *B. marginata* to seasonal fluctuations in its natural habitat was performed to provide information on the mechanisms used by this species to survive climatically stressful periods. Since the limits of a species' range are largely determined by seasonal extremes of climate (Sinclair 1980), particularly drought and cold in Tasmania, the water relations and frost tolerance of *B. marginata* at six sites along an altitudinal gradient were examined once a month, over a 14 month period.

The study was performed over a narrow geographical range, i.e. the straight line distance from the highest to the lowest site was only 8 km (Fig. 7.1). The sites were chosen for their proximity to one another to minimise genetic variability induced by population isolation. The drought and frost tolerance of the species was examined to determine whether the apparent survival of *B. marginata* in Tasmania during the cyclic glacial/interglacial events of the Pleistocene, and its subsequent success, is in some way related to the physiological plasticity and inherent genetic variability of this species.

Site Details

Mount Wellington - 1040 m altitude

Mount Wellington has an unpredictable climate. Frosts and snow have been recorded during every season and the mountain is often covered in mist and cloud. At this sub-alpine site *Banksia marginata* has colonised a dolerite boulder scree slope. Thus, in parts, the soil is very rocky and skeletal and over the drier season there is the potential for drought stress. The plants studied from this population appear to be healthy, mature, good seed producers, and range between 1.5-2 m in height.

Mount Nelson - 420 m altitude

Banksia marginata grows on a grassy bank on the mountainside. The site is unshaded, quite exposed to wind and with almost no over-storey canopy to shield the plants from frost. The plants studied are mature, healthy, good seed producers, and range between 1.5-2 m in height.

Ridgeway - 280 m altitude

Banksia marginata grows here within a dry sclerophyll, high light intensity heathland. Obvious morphological variation exists among members of this population, particularly with respect to leaf size and shape. The specimens studied are less than or equal to 1.5 m tall, with some verging on prostrate. Seed production is low and the soil is derived from a dolerite substrate.

Waterworks - 250 m altitude

The most striking feature of this roadside site is the abundance of *B. marginata* seedlings. The specimens studied appear to be healthy, mature, abundant seed producers, ranging between 2-3 m in height. The site has little or no over-storey canopy to offer protection to the plants during frost. The soil is of dolerite origin.

University - 120 m altitude

Banksia marginata grows within a gully here, which is often moist during winter. This is an environment of low light intensity since the plants are shaded by a *Eucalyptus* over-storey. Many of the leaves in this population had spots of chlorosis. The plants studied vary between 2-3 m in height.

Kingston Beach - sea level

Banksia marginata grows here on a very deep, sandy soil, close to the mouth of the Derwent River. The site is coastal and exposed to the sea breeze. The plants studied grow at the bottom of a hill and are subject to cold air drainage and significantly reduced sunlight hours over the winter. The population consists of mature seed producers, varying between 2-3 m in height.

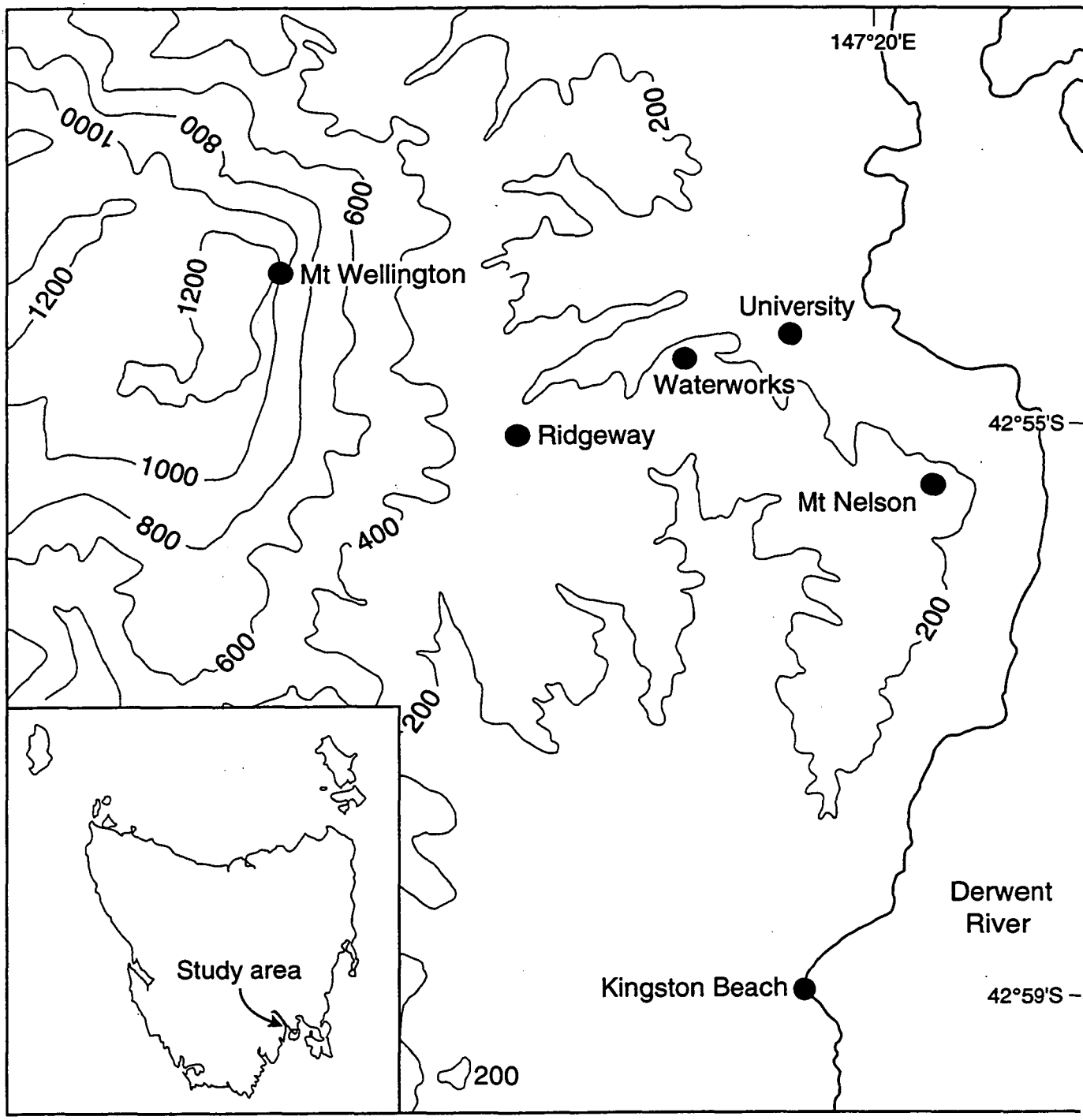

Figure 7.1. The cline study sites.

Methods and Materials

Frost

Once a month over a 14 month period, foliage was collected from each of the six sites along with the corresponding maximum and minimum temperatures and rainfall for Mount Wellington, Mount Nelson, University and Kingston Beach (see Table 7.1).

At the beginning of the experiment, five plants were randomly chosen at each site and labelled so they could be repeatedly measured on a monthly basis. At each site, shoots containing adult leaves of similar exposure, produced during the previous growing season, were removed with secateurs, labelled, and the cut ends were placed immediately into a bucket of water. On arrival at the laboratory, the shoots were left in water, covered with plastic and placed in a cool, dark room at 5°C overnight.

The next day, leaves selected for the experiment were rinsed twice in distilled water, to clean their surfaces. Five leaf discs (5 mm diameter) were taken per shoot per temperature treatment using a hole puncher. Each group of five leaf discs was placed in a separate glass vial with a drop of deionised water and a few crystals of AgI to prevent supercooling of samples during frosting (Hallam and Tibbits 1988; Read and Hill 1988).

A control was set up in which specimens were prepared exactly as above but were not frost treated. Methods from this stage follow those described in Chapter 4.

Data analysis

T50 values (the temperature at which 50% tissue damage occurs) were obtained by applying a probit transformation to the *It* data, (see Chapter 4).

Statistical analysis

The procedure PROC GLM in SAS (1988) was used to undertake an analysis of variance on the *T50* values with a statistical model that included term r for the fixed effects of site and month, the random effect of replication within month, and the interaction between site and month. The significance of the differences of the estimated means for each site was tested using Tukey's method to highlight any significant differences among the sites.

Water Relations

The pressure volume curve method of Mooney and Davis (1986) was followed to determine the monthly water relations of *B. marginata* from four sites (Mount Wellington, Mount Nelson, University and Kingston Beach). Shoots of adult foliage were excised from the terminal ends of branches. To prevent air entry into the xylem, cut shoot ends were immediately sealed with wet cotton wool, placed in a bucket of distilled water and excised further up the stem. After excision, the specimens were labelled and the cut end placed in distilled water (Mooney and Davis 1986). Specimens were covered with a plastic bag and placed in a cool dark room at 5°C overnight to achieve turgidity.

The next day the shoots were blotted dry and the turgid weight and corresponding water potential were obtained using a Scholander style pressure bomb. The *PVC* method described in Chapter 4 was used to examine the water relations of the different populations of *B. marginata*.

Data analysis

The water relations data was analysed using "TISWAT.BAS", the computer program of Davie *et al.* (1993) to obtain the osmotic potential (π_O) at full turgor, bulk modulus of elasticity of near turgid plants (ε), apoplastic water content (R^*a), relative water content and water potential at turgor loss.

Statistical analysis

The procedure PROC GLM in SAS (1988) was used to undertake an analysis of variance on the π_0 at full turgor, ε, R^*a, relative water content and water potential at turgor loss with a statistical model that included term r for the fixed effects of site and month, the random effect of replication within month, and the interaction between site and month. The significance of the differences of the estimated means for site was tested using Tukey's method.

Results

Frost

All populations, regardless of altitude and time of year, were inherently frost tolerant, with the least negative *T50* value being -6°C for Kingston Beach in July 1993 (Fig. 7.2c). There was a trend for all sites to have their most negative *T50* over the summer and/or autumn of 1994, with good frost tolerance also extending into the cooler winter months of 1994 (Fig. 7.2a, 7.2b and 7.2c). The frost tolerance of *B. marginata* for all sites actually improved over the summer, possibly as a result of hardening in response to drought stress.

It is clear that all populations of *B. marginata* were able to vary their frost tolerance considerably from month to month (Fig. 7.2a, 7.2b and 7.2c), thus highlighting the physiological plasticity of this species. For example, Tukey's test indicates significant differences ($P < 0.05$) in the monthly level of frost tolerance at each site, for at least some of the months studied (see Tukey's test in Appendix 3).

Table 7.2 represents the mean frost tolerance for each site, and indicates a trend for improved frost tolerance with altitude. Table 7.2 indicates that Mount Wellington *B. marginata* was noticeably more frost tolerant than the other populations, but the lowest altitude, least frost tolerant Kingston Beach population was not very different from the higher altitude Waterworks population. *Banksia marginata* from Mount Nelson and Ridgeway were more frost tolerant than the lower altitude Kingston Beach, University and Waterworks populations. While these results imply only a weak correlation

between frost tolerance and altitude, there were significant differences ($P < 0.05$) in frost tolerance among the populations (see analysis of variance table in Appendix 3).

In summary, Table 7.2 and Tukey's test highlight the physiological variation among the different populations of *B. marginata* across the sites. This result, combined with the demonstrated physiological plasticity of this species (Figs 7.2a, 7.2b and 7.2c), may contribute to its successful occupation of a wide range of habitats in Tasmania.

Water Relations

These results are complex and only the most significant aspects are highlighted. Figure 7.3a and 7.3b shows fluctuations in the monthly π_o of *B. marginata* from four sites, however, analysis of variance indicates that monthly variation is only significant ($P < 0.05$) for the Kingston Beach and Mount Nelson populations. Table 7.3 and Fig. 7.3a and 7.3b indicate that the Mount Wellington population of *B. marginata* usually had the least negative π_o at full turgor.

The relative water content at turgor loss varies among the sites (Fig. 7.4a and 7.4b). Site differences are most clearly demonstrated in Table 7.4, where the means of monthly values for relative water content at turgor loss are displayed. Table 7.4 indicates that the Mount Wellington population lost turgor at lower *RWC* than *B. marginata* from the other sites. In addition, analysis of variance indicates that *B. marginata* from Kingston Beach and Mount Nelson were able to significantly vary ($P < 0.05$) the *RWC* at which they lost turgor for some of the months (Fig. 7.4a and 7.4b).

The results for the water potential at turgor loss also varied among the sites (Fig. 7.5a and 7.5b). The differences among the sites are also more clearly demonstrated in Table 7.5, where means of monthly values for water potential at turgor loss are displayed. Table 7.5 indicates that the Mount Wellington population lost turgor at higher water potentials than the other populations. Both the Mount Wellington and Kingston Beach populations lost turgor at noticeably higher water potentials than the University and Mount Nelson populations. In addition, Tukey's test indicates that *B. marginata* from Mount Wellington and Mount Nelson were able to significantly vary ($P < 0.05$)

the water potential at which they lost turgor for at least some of the months (Fig. 7.5a).

Of the populations studied, Mount Wellington had noticeably lower ε and higher R^*a values from month to month than the other populations (Fig. 7.6a, 7.6b, Table 7.6 and 7.7a, 7.7b, Table 7.7 respectively). Figure 7.6a and 7.6b indicate fluctuations in the ε value of *B. marginata* from all sites, although, according to the analysis of variance, only the populations from Mount Wellington and Mount Nelson (the sites of highest altitude) demonstrated significant differences ($P < 0.05$) in ε for certain months.

Although Fig. 7.7a and 7.7b indicate monthly variations in R^*a for all sites, none of the populations showed significant monthly differences ($P > 0.05$).

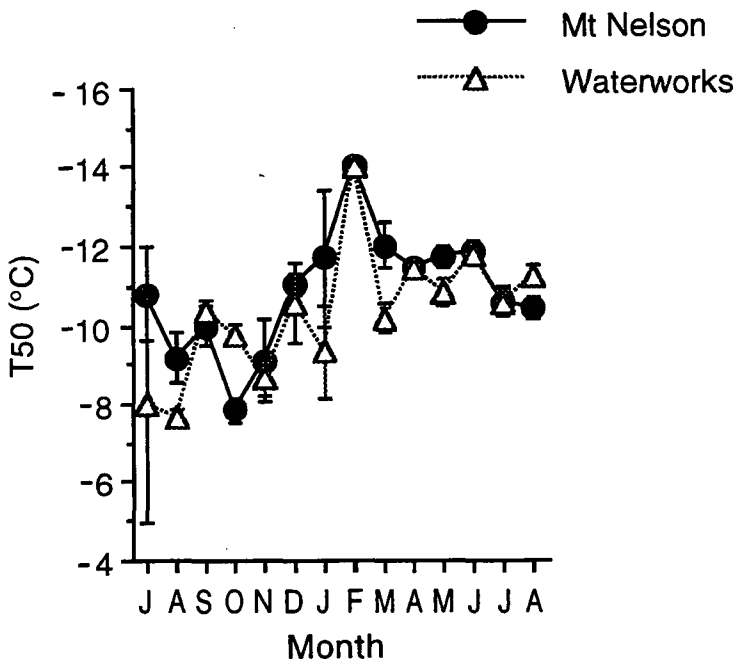

Figure 7.2a A comparison of the frost tolerance (T50 °C at 50% tissue damage) of *Banksia marginata* from Waterworks and Mount Nelson. Standard error bars are shown.

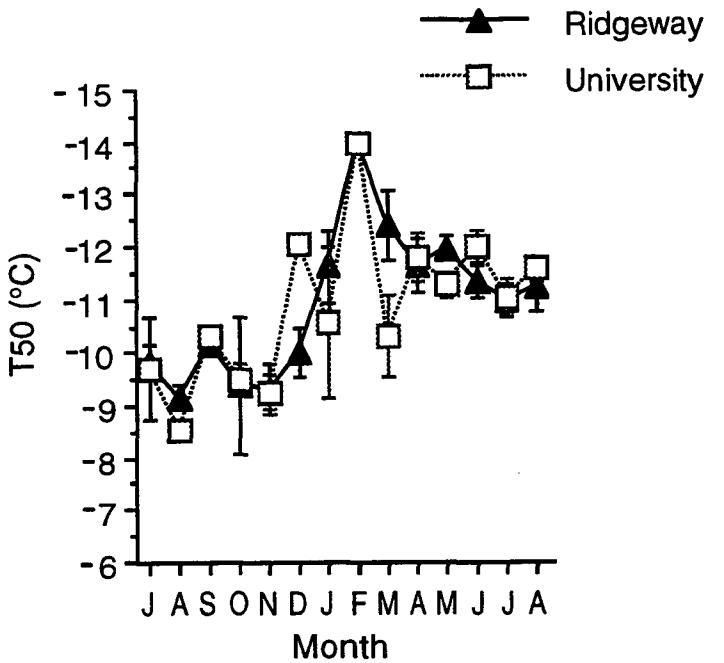

Figure 7.2b. A comparison of the frost tolerance (T50 °C at 50% tissue damage) of *Banksia marginata* from University and Ridgeway Reserve. Standard error bars are shown.

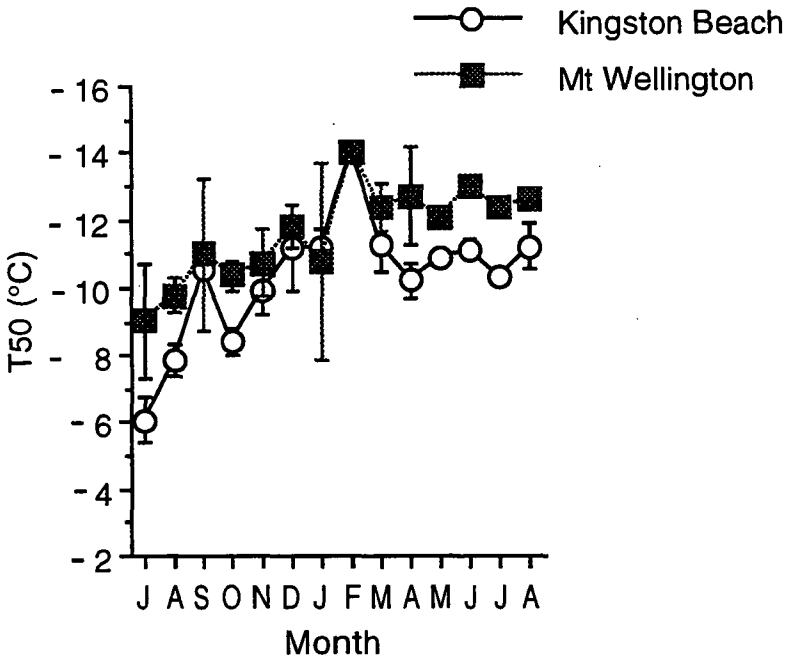

Figure 7.2c. A comparison of the frost tolerance (T50 °C at 50% tissue damage) of *Banksia marginata* from Mount Wellington and Kingston Beach. Standard error bars are shown.

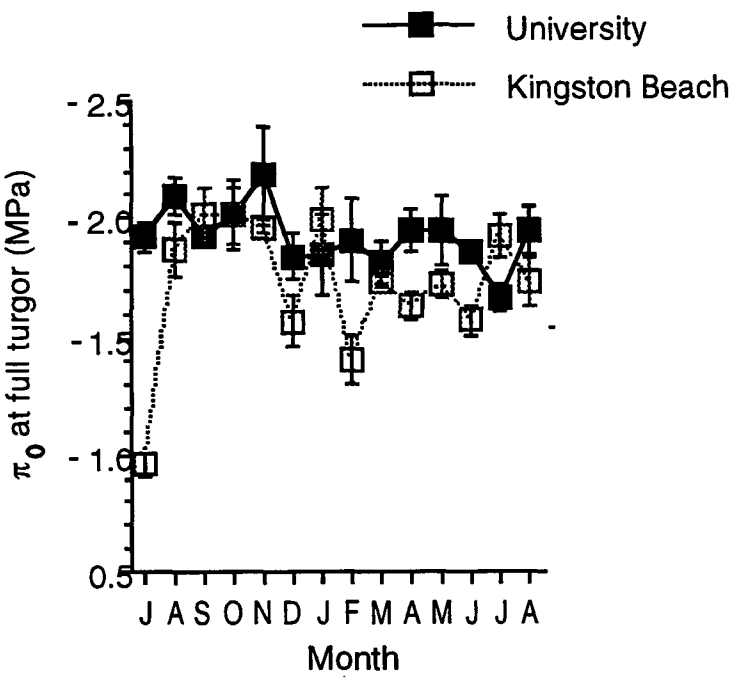

Figure 7.3a. The monthly osmotic potentials *(π_O)* of *Banksia marginata* from Kingston Beach and University. Standard error bars are shown.

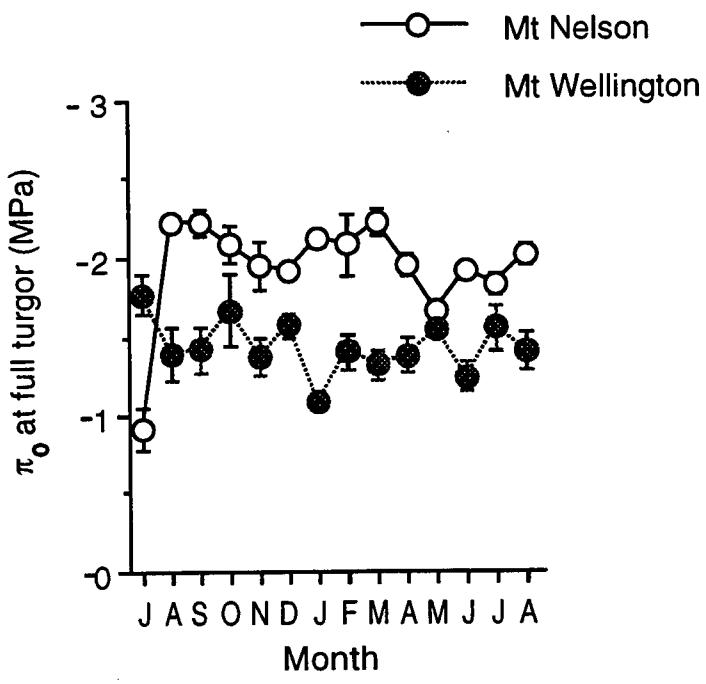

Figure 7.3b. The monthly osmotic potentials *(π_O)* of *Banksia marginata* from Mount Wellington and Mount Nelson. Standard error bars are shown.

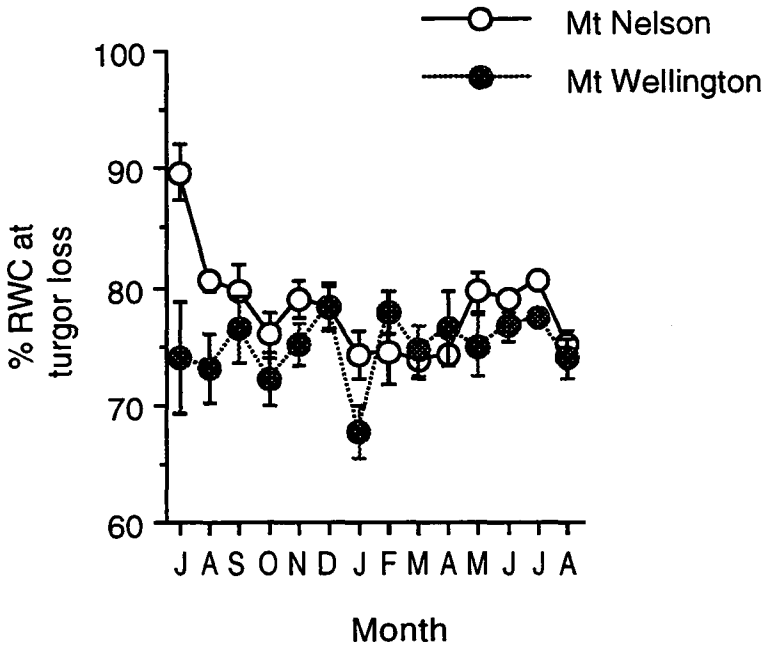

Figure 7.4a. The monthly changes in relative water content at turgor loss of *Banksia marginata* from Mount Wellington and Mount Nelson. Standard error bars are shown.

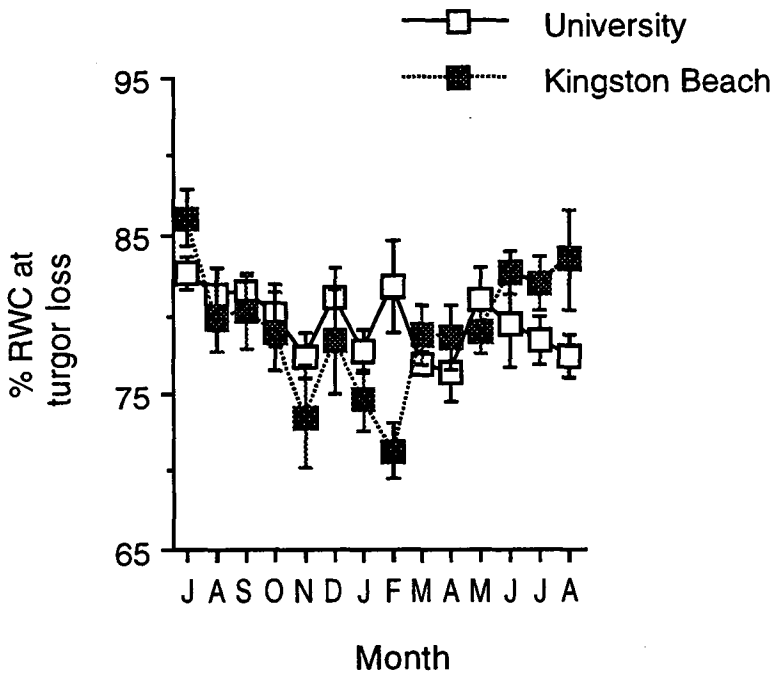

Figure 7.4b. The monthly changes in relative water content at turgor loss of *Banksia marginata* from Kingston Beach and University. Standard error bars are shown.

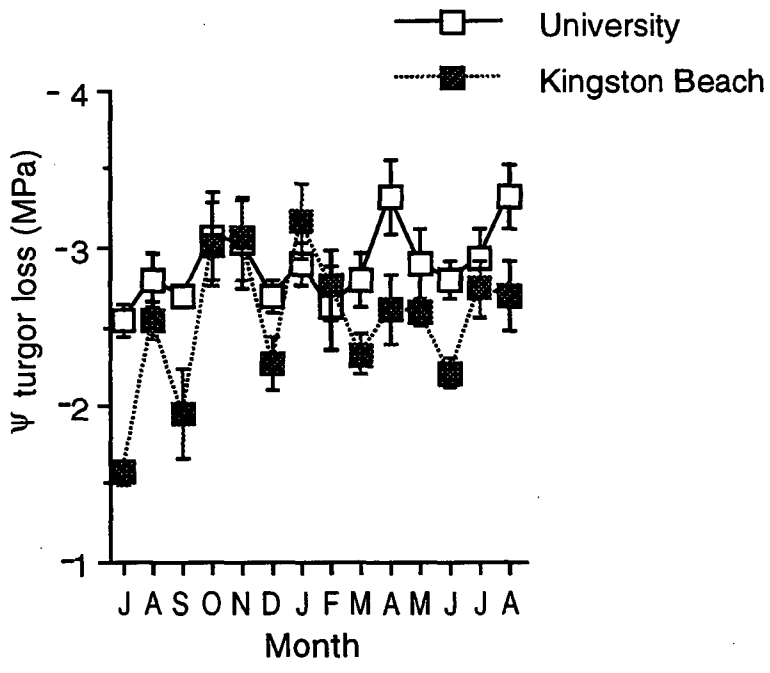

Figure 7.5a. The monthly changes in water potential (ψ) at turgor loss of *Banksia marginata* from Kingston Beach and University. Standard error bars are shown.

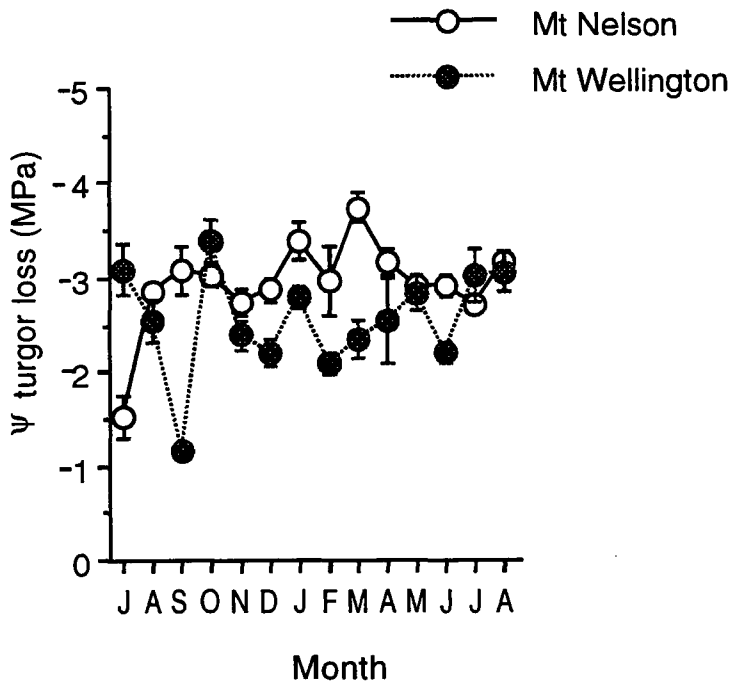

Figure 7.5b. The monthly changes in water potential (ψ) at turgor loss of *Banksia marginata* from Mount Wellington and Mount Nelson. Standard error bars are shown.

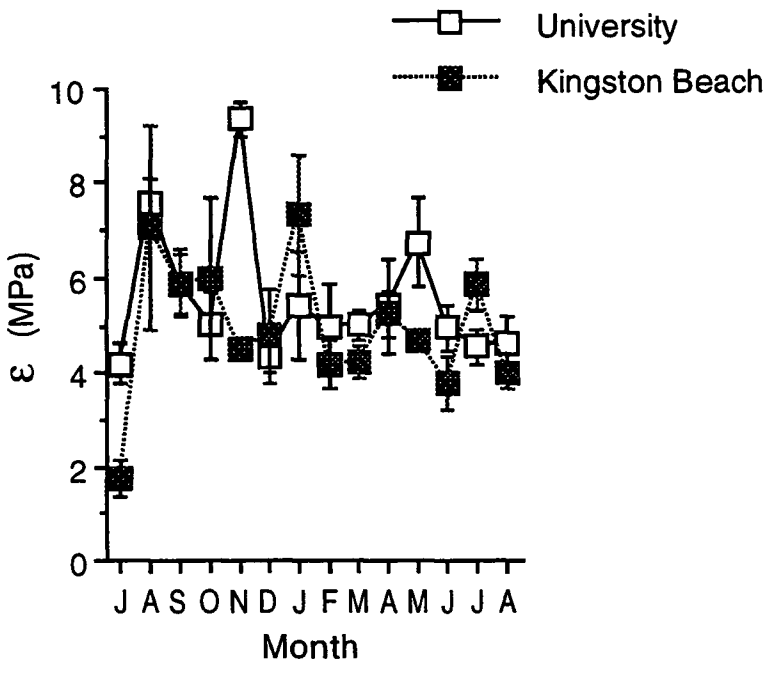

Figure 7.6a. The monthly changes in bulk elastic modulus *(ε)* of *Banksia marginata* from Kingston Beach and University. Standard error bars are shown.

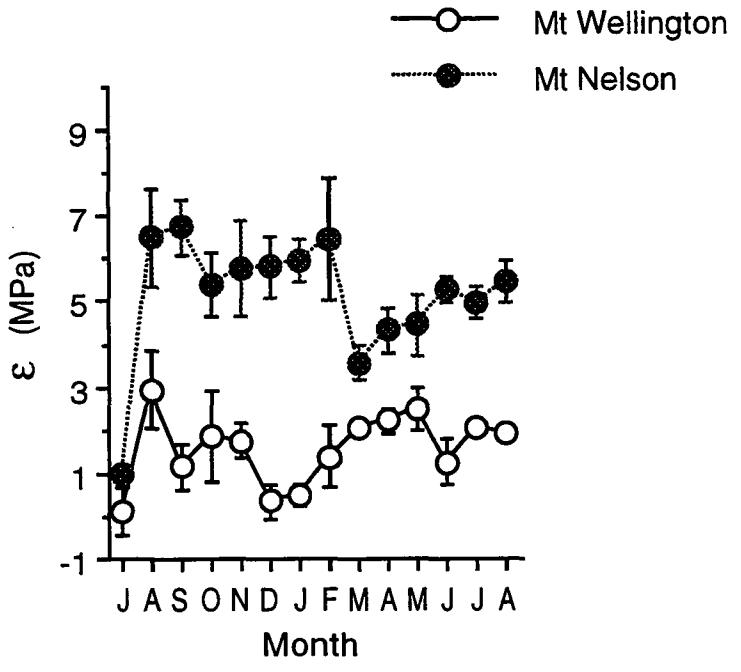

Figure 7.6b The monthly changes in bulk elastic modulus *(ε)* of *Banksia marginata* from Mount Wellington and Mount Nelson. Standard error bars are shown.

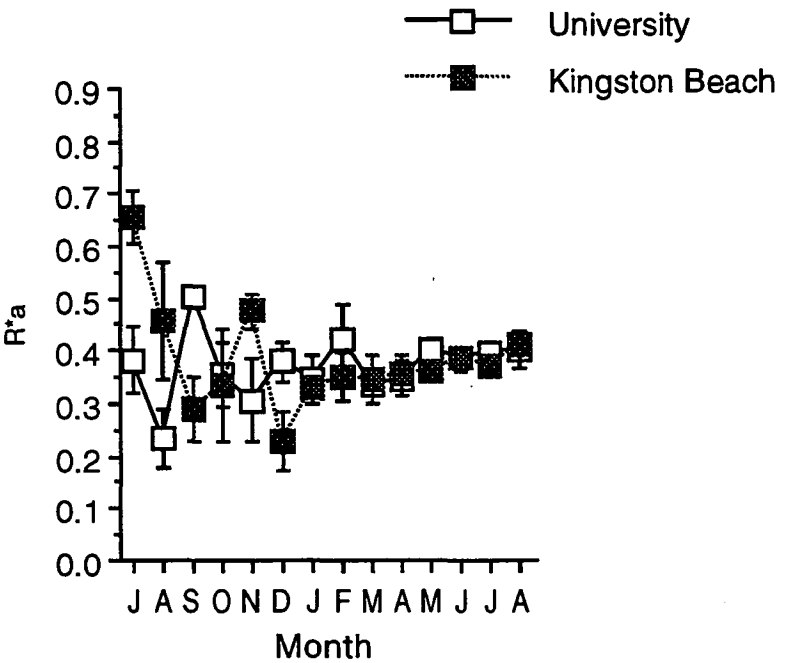

Figure 7.7a. The monthly changes in apoplastic water content *(R*a)* of *Banksia marginata* from Kingston Beach and University. Standard error bars are shown.

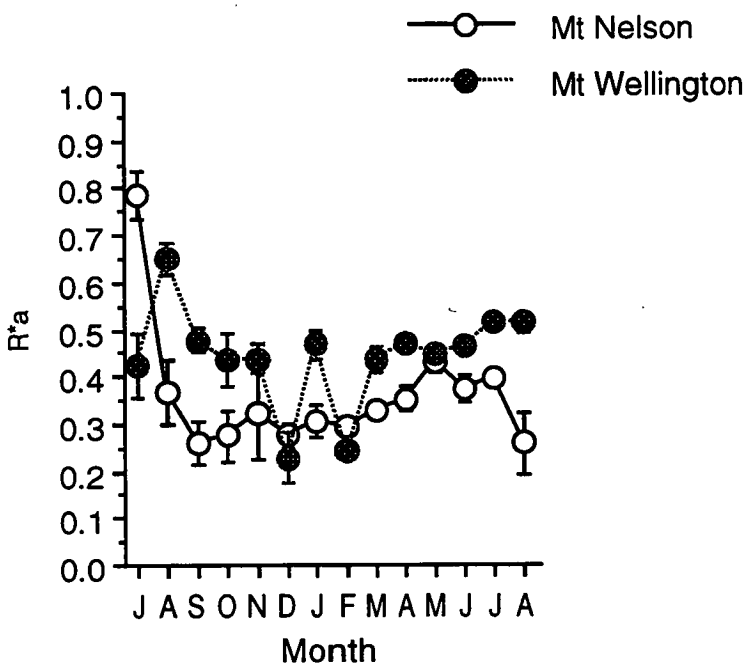

Figure 7.7b The monthly changes in apoplastic water content *(R*a)* of *Banksia marginata* from Mount Wellington and Mount Nelson. Standard error bars are shown.

Table 7.1 Monthly rainfall (mL)and maximum and minimum temperatures (°C) for Mount Wellington, Mount Nelson, University and Kingston Beach.

Site	Temperature (T) and rainfall (RF)	July 1993	Aug	Sept	Oct	Nov	Dec	Jan 1994	Feb	Mar	April	May	June	July	Aug
Mt Wellington	Maximum T				20	16	26.5	20	31	23	22.5	19	11	10	12
Mt Wellington	Minimum T				-3.8	-2	-1	0	0.5	0.5	-0.5	-2	-2.5	-4.5	-5
Mt Wellington	Monthly RF	99.2	83.8	22.2		370	1350	>2000	360	470	1000	510	1475	800	1900
Mt Nelson	Maximum T			17	22	21	32	26.5	38.5	27.5	25.5	23	20	15	14
Mt Nelson	Minimum T			1	0	1	5	5	5	5	3	5	2	0.5	1
Mt Nelson	Monthly RF					280	960	>2000	310	200	600	215	825	460	775
University	Maximum T			22	25.5	26	36	30	41	32.5	30	26.5	20	17	14
University	Minimum T			2	2	4.5	6	5.6	9	5	2.5	-0.5	2	-0.5	-2
University	Monthly RF					200	660	>2000	240	230	525	170	870	450	880
Kingston Beach	Maximum T				23	21	32	22	37	27	24	25	18	15	14.5
Kingston Beach	Minimum T				1.5	4.5	5	7	6.5	6	5.5	1	0.5	1	-0.5
Kingston Beach	Monthly RF	5.4	6	0		105	750	>2000	275	200	575	215	800	335	850

Table 7.2. A comparison of the means of the monthly frost tolerance (*T50* °C) for *Banksia marginata* from each site.

Site	*T50*	Standard Error
Mount Wellington	-11.25	0.33
Mount Nelson	-10.58	0.36
Ridgeway Reserve	-10.64	0.31
Water Works	-9.95	0.36
University	-10.53	0.32
Kingston Beach	-9.90	0.45

Table 7.3. A comparison of the means of the monthly osmotic potentials (π_O) MPa of *Banksia marginata* at full turgor.

Site	π_O	Standard Error
Mount Wellington	-1.44	0.05
Mount Nelson	-1.93	0.09
University	-1.94	0.03
Kingston Beach	-1.72	0.08

Table 7.4. A comparison of the means of the monthly relative water contents at turgor loss for *Banksia marginata.*

Site	*% RWC*	Standard Error
Mount Wellington	75.16	0.80
Mount Nelson	78.18	1.21
University	79.42	0.64
Kingston Beach	77.92	1.22

Table 7.5. A comparison of the means of the monthly water potentials (Ψ) MPa at turgor loss for *Banksia marginata.*

Site	Ψ	Standard Error
Mount Wellington	-2.50	0.15
Mount Nelson	-2.92	0.14
University	-2.87	0.05
Kingston Beach	-2.54	0.15

Table 7.6. A comparison of the means of the monthly bulk elastic modulus of elasticity *(ε)* MPa of near turgid *Banksia marginata.*

Site	ε	Standard Error
Mount Wellington	1.48	0.26
Mount Nelson	5.60	0.36
University	7.62	0.17
Kingston Beach	6.41	0.14

Table 7.7. A comparison of the means of the monthly apoplastic water contents *(R*a)* of fully turgid *Banksia marginata.*

Site	*R*a*	Standard Error
Mount Wellington	0.4270	0.028
Mount Nelson	0.3680	0.039
University	0.3717	0.016
Kingston Beach	0.3827	0.027

Discussion

Drought and frost are important forcing features in natural selection, and plant survival of glacial events will be largely dependent on a species' capacity to tolerate both of these stresses. Even today, the capacity to tolerate drought and frost is of great importance in Tasmania, where summer drought and winter frost are common events, and where out of season frosts occur at all but extreme coastal sites (Langford 1965). Snowfalls may occur at any time of the year in the highlands and unusual pressure systems have resulted in snowfalls at sea level in southern Tasmania (Langford 1965) as observed in 1986, 1991 and 1994. Furthermore, it is often the uncommon, though severe frost and drought events which cull less vigorous plant species. For example, severe and growing season frosts have reputedly prompted changes in species dominance in stands of *Eucalyptus* (Costin 1954; Burke *et al.* 1976; Davidson and Reid 1985).

The cool, often frosty nature of Tasmania's past and current climate may account for the good frost tolerance of the populations of *B. marginata* examined, regardless of altitude and season (Figs 7.2a, 7.2b, 7.2c). Similar levels of frost tolerance have been observed in the winter hardened Tasmanian endemics *Nothofagus cunninghamii, Phyllocladus asplenifolius* and *Eucryphia lucida* from 700 m above sea level (Read and Hill 1988), e.g. for August 1985, *T50* values were -12.5, -8.7 and -10.4°C respectively. These results however, are not directly comparable to those obtained in this study since the species were hardened in different years.

Larcher and Bauer (1981) have suggested that the distributional limits of plant species is more likely to be a function of long term climatic stress, such as that caused by a glaciation, than by infrequent, severe frost events. Indeed, some species are more adapted to past conditions than existing ones (Blake and Jordan 1993), particularly species which have a conservative genome. For example, it has been suggested by Read and Hill (1988) that the high frost tolerance of *N. cunninghamii* may be a retained feature reflecting its evolutionary and climatic history and having facilitated its survival during the Late Tertiary cooling and glaciations of the Pleistocene. Consequently, it is also possible that the relatively high frost tolerance displayed by *B. marginata* is a retained feature, having resulted from the

adaptation of this species to the cooler, drier glacial climates of the Pleistocene. As an example of how much cooler the Last Glaciation was, Leopold (1967) indicates temperatures during the glacial maximum were on average 8-10°C cooler in the Snowy Mountains area of south-eastern Australia than at present.

However, it is also possible that *B. marginata* is a relatively recently evolved species. As indicated previously, Tasmania has had a complex climatic history with the glacial and interglacial dynamics perhaps providing the stimulus for speciation. Glaciations have reportedly been the stimulus for speciation in some taxa (Mielke 1989), e.g. the Asteraceae (Leopold 1967).

The water relations results presented here are more complex than the frost results, with *B. marginata* (see Tukey's test in Appendix 3) from all sites, except the University, showing significant differences ($P < 0.05$) for certain months, for at least some of the water relations' aspects examined. The stability of the water relations of the University population may be a function of the more stable, closed canopy environment occupied by *B. marginata* at this site, compared to the other sites, which are more open and exposed. According to Mooney (1980), metabolic plasticity is only obvious in plants which occur in naturally open and disturbed habitats, and in this case, although the monthly water relations were stable (Figs 7.3a, 7.4b, 7.5a, and 7.6a) there were significant ($P < 0.05$) variations in the monthly frost tolerance of the University population (Fig. 7.2b).

The water relations and frost results together highlight the physiological plasticity of *B. marginata*, and may partly contribute to its success over a wide habitat range. Many other Tasmanian species however, also have the capacity to differ their frost hardiness with season, e.g. *Eucalyptus* spp. (Hallam 1986; Tibbits and Reid 1987), *Atherosperma moschatum, Phyllocladus aspleniifolius, Eucryphia lucida, Nothofagus cunninghamii* and *Athrotaxis selaginoides* (Read and Hill 1988). Indeed, although the capacity to alter frost tolerance with season is a common phenomenon in many cool temperate forests, it must be stressed that the majority of vascular plant species in Tasmania have much more restricted distributions than *B. marginata*. *Banksia marginata* is undoubtedly one of the more successful species in Tasmania as is evident from its occupation of a wide range of habitats. For comparison, the Tasmanian endemics, *P. aspleniifolius, A.*

selaginoides and *E. lucida* are generally restricted to regions of high rainfall (Curtis and Morris 1981).

This study also highlights the difficulty associated with interpretation of plant physiology results obtained from along an altitudinal gradient in a region where weather conditions are so variable and extreme. For example, the months of greatest frost tolerance in *B. marginata* do not necessarily coincide with winter and early spring as might be expected and has been observed in *Eucalyptus* species (Davidson and Reid 1987). For all sites except Mount Nelson, there was a trend for the least negative *T50* values (most damage) to occur over July and/or August of 1993 (Figs 7.2a, 7.2b and 7.2c). Although some climatic data for that time is missing, by Tasmanian standards, the winter of 1993 was relatively mild. Additionally, the months with the most negative *T50* values (least damage) often occurred over summer and early autumn (Figs 7.2a, 7.2b and 7.2c). No *T50* values were recorded in February for any of the populations at temperatures as low as -14°C. Monthly rainfall for all sites for February did not exceed 360 ml. Indeed, the months of summer (with the exception of January) and autumn were comparatively dry (Table 7.1) and thus the low *T50* values observed for these months may be partly due to populations of *B. marginata* hardening to the dry conditions. As an example, average autumn rainfall for Mount Wellington was 660 ml compared to 1391 ml for winter. For example, Vasil'yev (1961), Yamada (1989) and Nelson *et al.* (1993) have all observed improved frost tolerance in certain species exposed to drought stress.

Parallels between the physiological mechanisms of frost and drought tolerance have long been recognised in many plant species (Siminovitch and Briggs 1953; Sakai 1959; O'Neil 1983; Doi *et al.* 1986; Valentini *et al.* 1990) including changes in π_O in response to both drought and freezing stress (Levitt 1972). Osmotic adjustment results in the active accumulation of solutes within cells in response to drought (Bewley and Krochjove 1982; Turner 1986; Virk and Singh 1990) and cold stress. For example, in a study of *Cryptomeria japonica*, the most negative π_O values were recorded over the coldest part of the study period (Doi *et al.* 1986). Similarly, Valentini *et al.* (1990) found the most negative π_O of *Eucalyptus regnans* occurred over the winter part of their study and O'Neil (1983) has observed osmotic adjustment during cold acclimation in *Frageria virginiana*.

Osmotic adjustment facilitates turgor maintenance (Turner and Jones 1980; Turner 1986) and cell membrane integrity, which are essential to the maintenance of normal cell metabolic processes (Hsiao 1973). Osmotic adjustment has also been observed to delay ice crystal formation in plants under cold stress (Larcher 1980). Consequently, the capacity to undergo osmotic adjustment whether in response to drought or cold stress would be expected to significantly improve a species' chance of surviving drought and frost during and outside of glacial events.

Of the four populations examined for water relations, only those from Mount Nelson and Kingston Beach demonstrated the capacity for osmotic adjustment (Figs 7.3a and 7.3b and Tukey's test). With respect to Mount Nelson, July 1993 was the only month in which the π_o differed significantly ($P < 0.05$) from the other months. Thus, except for the Kingston Beach, π_o probably has only a minor role in assisting the frost and drought tolerance of the populations of *B. marginata* studied.

It is probable that at least for the Mount Wellington population, low ε values and high *R*a*, rather than osmotic adjustment, assist in its frost and drought tolerance (Figs 7.3b, 7.6b, 7.7b, Tables 7.3, 7.6 and 7.7). For example, the Mount Wellington population generally had:
(*1*). the greatest frost tolerance (Fig. 7.2c, Table 7.2).
(*2*). the least negative π_o (Fig. 7.3b, Table 7.3).
(*3*). the most elastic ε cells (Fig. 7.6b, Table 7.6), and
(*4*). the highest *R* a* (Fig. 7.7b, Table 7.7).
The results for π_o in this study are contrary to the negative correlations found between frost tolerance and π_o by Doi *et al.* (1986), O'Neil (1983) and Valentini *et al.* (1990). In other words, they found the more negative the π_o of a species, the greater its frost tolerance.

Indeed, different plant species and different populations of the same species have different ways of dealing with cold stress. According to the terminology of Larcher (1980) the Mount Wellington population is freezing tolerant. In freezing tolerant plants, intracellular ice crystal formation is avoided through the displacement of symplastic water to the apoplast (Larcher 1980), hence the comparatively high *R*a* for this population (Fig. 7.7b, Table 7.7). High *R*a* have also been observed in plants which have been pre-conditioned to water stress (Blake and Tschaplinksi 1992). The flow of water into the

apoplasm has been reported to protect living cells from damage by protecting them from sudden water loss during subsequent dehydration (Blake and Tschaplinksi 1992). Apparently, in Australia, most plants living in frost prone sites tend to delay intracellular ice crystal formation through the lowering of their π_O (Larcher 1980) rather than by increasing their *R*a*. However, the significantly smaller ($P < 0.05$) ε of the cells observed for the Mount Wellington population of *B. marginata* (Table 7.6) should enable it to tolerate symplastic dehydration, whether it is the result of drought or cold stress.

Low values of bulk elastic modulus (elastic cells) enable cells to shrink in volume and at the same time facilitate turgor maintenance. Elastic adjustment results from modifications in the cell walls which make them more elastic, thereby facilitating tissue shrinkage during dehydration (Blake and Tschaplinksi 1992). Joly and Zaerr (1987) have suggested that elastic shrinkage rather than osmotic adjustment may be of more importance for drought resistance, particularly in repeatedly stressed woody plants. Mount Wellington is the most climatically stressful of the sites studied with respect to cold. In addition, the skeletal nature of the scree slope substrate on which the population occurs makes for a particularly dry site, especially over the summer, even though rainfall is comparatively high.

In July 1993, Mount Wellington and Mount Nelson populations of *B. marginata* had their most elastic cells (Fig. 7.6b). The Mount Nelson population also had its highest *R*a* for this month (Fig. 7.7b) which was a comparatively dry month (see Table 7.1). However, the Mount Wellington population of *B. marginata* had highly elastic cells for the cool, though comparatively wet months of December and January (Fig. 7.6b and Table 7.1). These results suggest that reductions in ε can be stimulated by both cold and water stress at this site.
Little information could be drawn from the relative water content and water potential at turgor loss (Figs 7.4a, 7.4b, 7.5a and 7.5b) respectively. However, at least for the relative water content at turgor loss, the results are more meaningful when averaged over the months (Table 7.4). These averaged results indicate that the Mount Wellington *B. marginata* population is able to delay turgor loss to lower *RWC* levels than *B. marginata* from the other sites. Thus, lower relative water contents at turgor loss, higher *R*a*, and overall greater cell elasticity indicate that the Mount Wellington population of *B. marginata* is more desiccation tolerant than *B. marginata*

from the other sites. Desiccation tolerance is beneficial to plants exposed to drought and cold stress and may have helped certain populations of *B. marginata* to survive Pleistocene glacial events.

Analysis of variance indicates significant differences ($P < 0.05$) among the populations for frost tolerance and every aspect of the water relations except *R*a* (Figs 7.3-7.7, Tables 7.2-7.7, Appendix 3). These results thus highlight significant ($P < 0.05$) physiological differences among *B. marginata* across the sites examined. The significant variation observed among the populations may:

(*1*). reflect the diverse gene pool associated with this species and/or,

(*2*). reflect its physiological plasticity.

To conclude, this study highlights:

(*1*). some of the physiological mechanisms used by *B. marginata* to survive climatically stressful sites,

(*2*). its physiological plasticity and,

(*3*). the physiological variation of this species over a narrow geographical range.

The apparent survival of glacial events by *B. marginata*, its subsequent invasion of a wide range of niches, and its current occupation of a diverse range of habitats is therefore not surprising and may be the result of a combination of all of the above factors. Mount Wellington is the most climatically stressful of the sites examined and this population is clearly the most hardy with respect to frost, with its drought tolerance perhaps being a function of its good frost tolerance or vice versa. The interrelatedness of various physiological factors which operate in plants in response to more than one environmental stress (Roberts *et al.* 1980) has been well demonstrated here.

From an historical point of view, during the Last Glacial when the tree line was forced to lower altitudes due to cold stress, it is likely that only the most hardy populations of *B. marginata* would have survived. Survival would have largely depended on the ability of this species to retract to refugia, i.e. to sites where climatic conditions were more suitable to survival. With the return of the warmer conditions of the current interglacial, *B. marginata* is likely to have expanded out from its refugia perhaps combining its drought and cold tolerance with physiological plasticity to invade any available

niches. Furthermore, its morphological variability has been well documented and many times has resulted in its misclassification into multiple species (Salkin 1979). The inherent morphological variability of this species and the significant differences ($P < 0.05$) between the sites for the frost and water relations experiments suggests the association of a diverse gene pool with this species, a striking result considering the narrow range over which it was studied.

An alternative argument is to suggest that the excellent drought and frost hardiness of this species, regardless of altitude and time of year, enabled it to expand its range actually during the Last Glacial, facilitating its invasion of any suitable niches made available to it through glacial plant extinctions.

An isozyme analysis of the genetic variability of *B. marginata* would complement this study and give greater insight into the success of *B. marginata* in Tasmania. A limited examination of this has been performed (see next chapter).

CHAPTER 8. GENETIC VARIATION AMONG TWO POPULATIONS OF *B. MARGINATA* ALONG AN ALTITUDINAL CLINE.

Introduction

The results from the previous chapter indicate significant differences among the populations of *B. marginata* examined along the cline for drought and cold tolerance over a 14 month period. The results from the previous chapter also demonstrate phenotypic plasticity in *B. marginata*. Auld and Morrison (1992) maintain that the capacity for plasticity enables species to occupy a wide range of different habitats without any changes occurring to the genome. By contrast, other successful species are able to achieve widespread distribution by possessing a sufficient degree of genetic variation (Auld and Morrison 1992).

It is evident from the results of the previous chapter that the success of *B. marginata* in Tasmania today is likely to be a combination of its capacity for physiological plasticity and the genetic diversity of the species. To date however, no attempt has been made to examine the intra-specific variation of *B. marginata* from a genetic perspective. An isozyme analysis was performed on two of the same field populations examined in the Cline. The populations examined were from the University Reserve and Mount Wellington. As indicated in the previous chapter, these two populations differed significantly from one another for the following aspects of their drought physiology: π_O at full turgor, relative water content at turgor loss, water potential at turgor loss, R^*a and ε. The University and Mount Wellington populations were only barely different for cold tolerance.

An isozyme analysis was performed to determine whether the two populations were genetically different. Indeed, although the isozyme genes and the genes which control drought and cold tolerance may be different, any difference in the isozymes of the two populations would imply that there could be a genetic basis to the physiological differences observed between the two populations.

Genetically fixed intra-specific variation in plants has been a noted response to many habitat factors: e.g. soil moisture, fertility and heavy metal

contamination, fertiliser enriched soils, snow melt, photo-period and temperature (Turkington and Aarssen 1984) as well as to a degree on wind exposure (Aston and Bradshaw 1966). Indeed, intra-specific variation is high in many species, with populations being differentiated spatially as ecotypes (Auld and Morrison 1992).

Materials and Methods

Two populations were examined, the University Reserve and Mount Wellington populations. These populations occupy climatically different sites that represent two distinct topographic and climatic regions, habitats and community associations.

Soft new shoots of the summer flush foliage were collected from 50 and 48 randomly selected plants of *B. marginata* from the University and Mount Wellington populations respectively. After collection, the foliage was placed in water, covered in plastic and stored in a cool room at 5°C before use. The isozyme analysis of this material was completed within two weeks of collection. These plants have been tagged for future reference. Refer to the previous chapter for site descriptions.

Electrophoresis

Isozyme analysis is a form of protein electrophoresis popularly used to highlight genetic diversity within and between different species and populations of plant and animal. Electrophoresis is a process whereby protein extracts containing enzymes are inserted into a gel matrix.

For this experiment, 10% W/V potato starch gels were prepared the day before the experiment. The next day, enzyme extracts were prepared by crushing young leaf material (~2-3) leaves with a mortar and pestle in 1.5-2.0 ml of 0.2 M pH 8 Tris extraction buffer (Table 8.1).

Table 8.1 Electrophoresis enzyme extraction buffer (modified from Wendel and Weeden 1989).

Stock solution (per 100 ml):
10 ml 0.2M Tris HCL buffer 8.1 pH
40 mg Polyvinyl pyrrolidine (PVP) (4000MW)
10 ml Glycerol
per 10 ml of stock add:
10 µl 2-mercaptoethanol (M-3148)
200 µl 25% Triton-X100 (T-6878)
10 mg BSA (Albumin bovine serum)
50 mg L-Ascorbic acid (A-7631)
20 mg Magnesium chloride

These extracts were absorbed into paper wicks 3x15 mm and loaded into horizontal starch gels (Wendel and Weeden 1989). A red dye marker was placed in the gel at the beginning and end of a line of extract wicks. After wick placement, the gels were placed at 5°C in either a standard, histidine or citrate buffer. An electrical current was applied underneath the gels for 10-15 minutes or until the red dye markers had moved 0.5 cm across the gel. After this, the wicks were removed, the gels were placed back into the buffer and the electrical current was run until the marker had moved ~10 cm (2.5-4.5 hours). At the end of this time, the gels were sliced horizontally and the slices were stained Table 8.2). After staining, the isozymes appeared as bands at different migration distances across the gel matrix (Brewbaker *et al.* 1968). Since enzymes are composed of uncharged sub-units it is mostly their net charge which will determine the distance migrated across the electrical gradient (Gottilieb 1977).

Initially, 15 enzyme specific stains (see Wendel and Weeden 1989, Table 3) were screened to determine which stains clearly and consistently produced polymorphic loci. A single locus was considered to be represented by a single zone of enzyme activity. Enzyme loci were numbered sequentially, starting with the most anodal called "1", and proceeding cathodally with

increasing numbers. A total of four putative diallelic loci were resolved clearly and consistently, these were: PER_1, $SKDH_1$, PGD_1 and PGD_2.

Table 8.2 Enzyme stains assayed for this study with the appropriate gel buffer system, enzyme commission number (E. C. no.) and abbreviation (abbrev, modified from Wendel and Weeden 1989). See Wendel and Weeden (1989) for stain recipes.

Enzyme	Abbrev	E. C. no.	Buffer
Alcohol dehydrogenase	ADH	1.1.1.1	H
Aspartate aminotransferase	AAT	2.6.1.1	S
Acid phosphatase	ACP	3.1.3.2	C/S
Aldolase	Aldo	4.1.2.13	H
Diaphorase	DIA	1.6.99.-	H
Esterase	EST	3.1.1.2	C
Glucose-6-phosphate isomerase	GPI	5.3.1.9	S
Glutamate dehydrogenase	GDH	1.4.1.2	S
Leucine aminopeptidase	LAP	3.4.11.1	H
Malate dehydrogenase	MDH	1.1.1.37	H
Malic enzyme	ME	1.1.1.40	C/S
Peroxidase	PER	1.11.1.7	S
Phosphogluconate dehydrogenase	PGD	1.1.1.44	H
Phosphoglucomutase	PGM	5.4.2.2	C
Shikimic dehydrogenase	SKDH	1.1.1.25	H/C

S, lithium borate/Tris citrate, pH 8.1 (Selander *et al.* 1971)
H, Histidine/Citric acid, pH 6.35 (Cardy *et al.* 1980)
C, Citrate/N-(3-aminopropyl)-morpholine, pH 6.1 (Clayton and Tretiak 1972).

Genetic diversity analysis

The computer package BIOSYS-1 was used to determine the genetic differentiation of the two populations (Swofford and Selander 1981). Intra-population variability was examined by allozyme frequencies, Hardy Weinburg analysis and Wright's (1965) fixation indexes to determine the amount of inbreeding. Allozyme frequencies were calculated from the genotypes observed in both parent populations. The allozyme frequencies were used to estimate probabilities of conformance to Hardy-Weinberg expectations and fixation indices for all polymorphic loci. Site differentiation was determined by a chi-square (χ^2) contingency test based on the expected and the observed heterogeneity within each population for the four polymorphic loci: $SKDH_1$, PGD_1, PGD_2, PER_1. In this case, it was expected that both sites would have the same number of heterozygote plants. See Appendix 5 for data used in the analysis.

Results

Four polymorphic loci, PER_1, $SKDH_1$, PGD_1 and PGD_2, were resolved and scored for both populations of *B. marginata*. These loci were all diallelic with PER_1 exhibiting very distinct band separation. Monomorphic loci were observed for several of the enzyme systems (ACP1, ME_1, MDH_1, G-6-$PGDH_1$ and $ALDO_1$). These were included in the total genetic variability analysis but excluded from any further analyses as they were not significant to the final results. Including the monomorphic data into further analyses would have no effect on the end results. Their inclusion would have been like including variables into a data set that were constant between experiments. Including them would have been like saying that those alleles have no heterozygosity and are not different between populations. Some bands were insufficiently distinct (DIA, EST and GPI) and could not be given the status of either polymorphic or monomorphic.

The allozyme frequencies were calculated for all polymorphic loci present in the two populations (Table 8.3). Most loci did not deviate from a Hardy-Weinberg equilibrium. However, two loci in the University population exhibited highly significant deviations from a Hardy-Weinberg equilibrium, PGD_1 and PGD_2. The PER_1 locus for the Mount Wellington population was barely significantly different from a Hardy-Weinberg equilibrium (Table 8.3).

Table 8.3 Allozyme frequencies and estimated probabilities of conformance to Hardy-Weinberg equilibrium for the Mount Wellington and University Reserve populations of *B. marginata*.

Locus	Allele	Mount Wellington	University
PER_1	A	0.775	0.777
	B	0.225	0.223
		p=0.044	p=0.827
		(n=51)	(n=47)
$SKDH_1$	A	0.275	0.649
	B	0.725	0.351
		p=0.218	p=0.664
		(n=51)	(n=47)
PGD_1	A	0.578	0.725
	B	0.422	0.275
		p=0.237	p=0.000
		(n=51)	(n=40)
PGD_2	A	0.204	0.432
	B	0.796	0.568
		p=0.354	p=0.002
		(n=49)	(n=44)

Table 8.4 Fixation indices (FI) for both parent populations from Mount Wellington and the University (1=fixed, 0=Hardy-Weinberg equilibrium, -1=Heterozygote excess).

Locus	Mount Wellington	University Reserve
PER_1	0.270	-0.042
$SKDH_1$	-0.181	-0.074
PGD_1	0.156	0.624
PGD_2	0.121	-0.482

The degree of inbreeding within a site is indicated by the fixation index (Wright 1965). The Fixation index (FI) is based on a comparison of the observed number of heterozygotes to the expected number under Hardy-Weinberg equilibrium. A large degree of inbreeding for a locus is represented by a value approaching 1. As the index tends to more than 1, more plants are fixed into extreme genotypes which are presented in this case by AA or BB. An index of -1 indicates more plants are heterozygous than expected at Hardy-Weinberg equilibrium. In this study, there are indications that both populations are not highly inbred since most fixation indexes are tending toward 0 (Table 8.4). For the locus PGD_1 however, the University population exhibited a high degree of inbreeding (F=0.624) while the PGD_2 locus indicates a heterozygote excess (F=-0.482).

A χ^2 test was conducted to determine the presence of genetic differentiation between the two parent populations (Table 8.5). The test was significant for three of the four polymorphic loci: $SKDH_1$ (p=0.0000), PGD_1 (p=0.045) and PGD_2 (p=0.0008). Genetic differentiation of the two populations was further supported when analysis combined all polymorphic loci (p=0.0000).

Table 8.5 Significance levels of χ^2 tests comparing allele frequencies between the University Reserve (Uni) and Mount Wellington (Well) Populations.

Comparison	PER_1	$SKDH_1$	PGD_1	PGD_2	Total
Uni*Well	0.9719	0.000	0.0405	0.0008	0.000

Discussion

The distinction between genetically fixed variation (population level variation) and phenotypic plasticity (or organisational level variation) has important implications for understanding the success of *B. marginata*.

Most importantly, the results for this experiment demonstrate that the Mount Wellington and University populations are significantly different from one another at a genetic level. Consequently, this implies that the significant differences observed in the drought and cold physiology among the two populations could very well have a genetic basis.

These results indicate genetic variation in populations of *B. marginata* over a short geographic distance. (i.e. < 8 km). The results suggest that the two populations may be discrete breeding entities rather than sub-populations of a larger interbreeding population.

Genetically fixed intra-specific variation in plants has been noted in response to many habitat factors, including soil moisture, fertility, heavy metal contamination, fertiliser enriched soils, snow melt, photo-period and temperature (Turkington and Aarssen 1984) as well as to a degree on wind exposure (Aston and Bradshaw 1966).

It is interesting that these two populations appear to be genetically distinct, especially since *B. marginata* is pollinated by animals. The results thus suggest that pollinator movement is limited between the two populations. This is unusual since most pollination is likely to be carried out by birds. Birds will frequently travel and pollinate over large distances (Carthew *et al.* 1988, Coates and Sokolowski 1992). This system would normally prevent populations from undergoing genetic drift.

However, considering the apparently limited gene flow between the two populations, it is possible the two populations have undergone genetic drift to some extent, although, there is no obvious "barrier" to pollen flow between the two populations. In Western Australia, an example of genetic drift has been recorded in *B. cuneata* where two populations separated by a salt

water river for a distance of less than 15 km have become genetically distinct from one another (Coates and Sokolowski 1992).

The selection pressures that affect a species' fitness vary considerably from site to site. The genetic differences observed between the two populations studied here might be due to different selection pressures operating at each site. Indeed, the Mount Wellington population occurs at a more climatically stressful site than the University population. The Mount Wellington population occurs on skeletal soil and is exposed to freezing temperatures for many months of the year.

At the extreme site of Mount Wellington, natural selection would have operated to select for those population members most physiologically tolerant of cold and drought stress. Rehfeldt (1993) maintains systematically distributed genetic variation along an environmental gradient undoubtedly arises from natural selection. Indeed, the overall physiology of the Mount Wellington population was generally significantly more frost and drought tolerant than the University (see previous chapter). Similarly, differences among the following populations of plant species separated along an altitudinal cline by the meters indicated have been recorded: *Pseudotsuga menziesii*-200 m (Rehfeldt 1989), *Pinus contorta*-250 m (Rehfeldt 1991) and *Carix occidentalis*-450 m (Rehfeldt 1982).

Monson *et al.* (1992) indicates that natural selection can overcome the potential for gene flow along environmental gradients, producing genetic differentiation within what appears to be one continuous plant population. Natural selection can work in two ways:
(1). when variation is genetically fixed, selection acts on specific traits or phenotypes and,
(2). when variation is due to phenotypic plasticity, selection acts directly on the capacity of an individual to respond developmentally to a varying environment (Diggle 1993).

Natural selection among different phenotypes is usually of little consequence to the future evolution of a population unless it produces genetic change (Strickberger 1976). The greater the genetic variability upon which selection for fitness may act, the greater the improvement in fitness. This principle is the basis for Fisher's fundamental theorem of natural selection, which states

in mathematical terms that the fitness of a population increases at a rate that is proportional to the genetic variability or genetic differences in fitness present in the population. It is easy to see that if a population in a particular environment were completely homozygous for all genes, selection for fitness in a changed environment would have little effect and produce no genetic improvement. Since populations do not always face constant environmental conditions, selection is not always for only one optimal genotype, and consequently genetic variability must be maintained for the population to survive (Strickberger 1976).

Grand fir *(Abies grandis)* occurs in the USA and is similar to *B. marginata* in that it occurs across a wide geographic range from below 300 m to 1180 m altitude (Chang and Cheng 1993). Five geographic races have been recognised (Fioles 1959 as cited in Chang and Cheng 1993). Chang and Cheng (1993) suggest that natural selection, genetic drift due to a reduction in population size after glaciation and restricted gene flow must have been important factors influencing the geographic differences of the species.

Monson *et al.* (1992) state that despite general knowledge that genetic differences occur across short distances within the range of a population, only a few studies (of which this is one) actually demonstrate the presence of physiological differences within a population of plant species.

CHAPTER 9 INTRA-POPULATION VARIATION WITHIN FIVE POPULATIONS OF *BANKSIA MARGINATA*

General Introduction

The water relations and frost results from the cline study indicate significant physiological variation among the different populations of *B. marginata* along the cline. In addition, the frost and water relations results for the seedlings of *B. marginata* from the eight Tasmanian populations also proved significant for certain of the hydroponic experiments. The levels of statistical significance were not as pronounced in the hydroponic experiments as in the cline. It is possible that the smaller differences observed among the populations of the *B. marginata* seedlings for some of the hydroponic treatments, is a consequence of the seedlings having all been grown under the same environmental conditions. This therefore raises the question of how much of the physiological variation observed in field populations of *B. marginata* is purely a phenotypic response to their physical environment?, i.e. a function of this species' genetic plasticity? However, regardless of the origin of the inter-population variation among this species, the fact that significant population differences do occur, provides an important clue behind the successful occupation of a wide range of habitats by *B. marginata.*

In addition to the obvious inter-population variation in the physiology of *B. marginata*, it is also interesting that for most, if not all populations of this species, at least in Tasmania, variation in tree morphology is obvious, even at a glance within populations. This variation in morphology is most obvious with respect to leaf shape, length, width, leaf tip, and cone size. Sometimes, within populations, a mixture of both prostrate and upright growth forms occur.

An obvious question to be addressed in this chapter is whether intra-population variation in the external morphology of *B. marginata* can be positively correlated with physiological variation, hence, providing another clue to the success of this species in Tasmania.

Differentiation within a localised population is dependent on the interplay between natural selection and gene flow; the more highly localised the

differentiation is, the higher and more directional the selection pressures need to be to counteract the gene flow (Grant 1981).

Methods

For this study, the importance of having considerable replication of sites and plants within sites cannot be stressed enough.

The choice of experiments and the study sites were determined by:
(1). distance from Hobart
(2). access to equipment and,
(3). access to technical assistance

Five populations of morphologically variable *B. marginata* were selected from around Hobart. Five populations was the estimated limit of what could be processed within a reasonable time frame, with the assistance available.

Frost and desiccation experiments were chosen to highlight any intra-population physiological variation in *B. marginata* from the five populations. The equipment for both of these experiments was readily available and the plant material could be collected the day before without any adverse effect on the results, as long as the shoots were kept well hydrated. Furthermore, the frost and desiccation techniques used should provide a reliable estimation of the field tolerances of this species. This is because it takes anywhere from a few days for cold and drought hardened plants to lose their tolerances once the stress has been relieved. According to DeHayes *et al.* (1990), and Hadley and Amundson (1992) a prolonged warm period (days) is necessary to produce a significant loss of cold tolerance. By contrast, photosynthesis and transpiration rates can change much more quickly than above (i.e. daily, hourly, minutely changes) in response to light intensity, water stress and diurnal changes. Therefore, to get a true representation of intra-population variation for these two factors, it would have been necessary to measure all plants, from all populations on the same day, at the same time, under the same light intensity and level of water stress. Even though many portable IRGAs (infra-red gas analysers) have in-built light sources that enable photosynthesis to be recorded for standard light intensities, unless the soil is at maximum field water storage capacity, it is almost impossible to standardise the level of drought stress each plant is under. Consequently,

because photosynthesis is largely affected by water stress, the value of determining a plant's rate of photosynthesis without knowledge of its Ψ would be uncertain.

The Population Sites

Six trees at each site were randomly selected and tagged. The same trees were used for both the frost and desiccation experiments.

Ridgeway 1 - The *B. marginata* population grows on a hill slope of about 30° within a heathy scrubland and at 390 m asl. The site is very open, with a high light intensity and a rocky substrate of dolerite origin. The plants studied ranged from 0.5-1.5 m in height. The last reported fire through the area was 10 years ago (P. McGlone pers. comm.). The site is subject to cold air drainage from Mount Wellington.

Ridgeway 2 - The *B. marginata* population grows on a 10-20° slope, at an altitude of about 300 m asl. The site is rocky, with a soil derived from a dolerite substrate. The *B. marginata* plants examined ranged in height from 3-5 m. The *Banksia* grow beneath a *Eucalyptus* sp. over-storey.

Southarm - This site is at sea level. The *B. marginata* population occurs within an old sand dune system and the shrub/trees selected were located between 300-400 m inland from the ocean. This is a high light intensity environment with minimal over-storey. The trees ranged from 0.5-4 m in height.

Primrose Sands 1 - This is a flat site at sea level. The substrate is sandy. The *B. marginata* plants examined ranged from 4-5 m in height.

Primrose Sands 2 - This site is about 200 m inland from the beach. The substrate is sandy and *B. marginata* ranged from 4-5 m in height. Both of the Primrose Sands sites are of high light intensity.

Field work

Three maximum and minimum thermometers were placed at each site three weeks prior to the experiments. The maximum and minimum temperatures for each site were recorded weekly until the time of the experiment (see Table 9.1). This was done to highlight any microhabitat temperature differences among the trees at the sites.

Table 9.1. Average weekly maximum and minimum temperatures (°C) at Primrose Sands (PR) sites 1 and 2, Southarm (SA) and Ridgeway Reserve (RW) sites 1 and 2, in 1994.

Site	Temperature	27/11/94	3/12/94	11/12/94
PR1	Max	33.25	24.33	41
PR1	Min	5.75	5	6.5
PR2	Max	32.3	28.25	35
PR2	Min	4.5	5.5	5
SA	Max	32.5	21.5	38.5
SA	Min	2.5	2.5	4.66
RW1	Max	33	28.16	41.33
RW1	Min	3	5.16	5.5
RW2	Max	30	27.16	32.5
RW2	Min	6	3.5	4.3

Pre-dawn Ψ for two shoots, per tree, per site were obtained the dawn prior to the laboratory experiments. Please refer to Chapter 4 for details of the method used.

The pre-dawn Ψ readings were taken to highlight any differences in the microhabitats of the individual trees which may have affected their desiccation and frost results. In this way, any differences in the tree-soil micro-habitat which may have influenced the intra-population variation in the *Banksia* physiology results could be identified (Table 9.2).

Table 9.2. Mean pre-dawn water potentials (MPa) of *B. marginata* from Primrose Sands (PR) sites 1 and 2, Southarm (SA) and Ridgeway Reserve (RW) sites 1 and 2, 1994.

Site	Ψ (MPa)	SE
PR 1	-0.270	0.029
PR2	-0.307	0.036
SA	-0.303	0.042
RW 1	-0.617	0.061
RW 2	-0.448	0.040

In preparation for the desiccation and frost experiments, two shoots were removed the day before the experiment from the southern side of the lower third of each tree, from each site. Each shoot was labelled as either a or b, i.e. two shoots were sampled from each tree. These shoots were placed in water, covered in plastic, returned to the lab and placed in a cool dark room at 5°C, overnight. In this way, the shoots were allowed to achieve full turgidity.

Laboratory Work

The next day, the fully turgid shoots were removed from the cold room. Leaves for the experiment were randomly selected from mature foliage produced during the previous growing season. This foliage was then washed in de-ionised water, cleaned and dried with tissues to remove any particulate matter which may have affected the results.

Please refer to Chapter 4 for a description of the desiccation procedure used.

The procedure described in Chapter 4 was used for the frost experiment with the following differences in sampling:
(1). two *B. marginata* shoots were sampled from six trees, from each of five sites and labelled the appropriate number of the tree (i.e. 1-6) and as either shoot a or b,
(2). four leaf discs were sampled per shoot i.e. two discs were taken from two separate leaves, to form one unit, as in the desiccation experiment.
In this study, a greater number of trees, and leaves per tree were sampled per site compared to experiments described in previous chapters. Unlike the previous experiments where the main objectives was to determine any differences in population response to stress, the aim of this experiment was to determine whether there was a correlation between leaf morphology and

physiology in the *B. marginata*. Consequently, a greater number of leaf and tree specimens were sampled than in previous experiments to increase the likelihood of finding a correlation. After data analysis (see later) it was apparent that many more sites and much more replication would be required to clarify the results.

As indicated in Chapter 6, the difference in the number of leaf discs used per shoot between the frost and desiccation experiments relate to the fact that there was not enough space to put any more than two discs per sample in the desiccators at the same time.

Morphology

A leaf morphometric Kurta XLC Digitiser System was used to obtain leaf and petiole lengths, and leaf width at the widest point of three leaves per shoot from six trees per site. The pointing device was moved over the relevant points to score the above characteristics. Although it was originally planned to examine many more morphological characteristics, it became apparent early on from the data analysis of the physiological characteristics, that it was unnecessary to go into any more morphological detail. The reasons for this will become apparent in the results section.

Data analysis

Probit Transformation

Probit transformations were performed on the *It* values from the desiccation and frost experiments, (see Chapter 4).

From the probit transformed desiccation and frost *It* it was possible to extrapolate the *T50* values (i.e. the *RWC* and temperature at which each plant examined succumbed to 50% tissue damage).

Statistical Analysis

Means were calculated for each trait (petiole length, leaf length, leaf width at the widest point, frost and desiccation tolerance) for each tree, and for each site. Pearson's correlations were calculated for each combination of a physiological trait with a morphological trait (SAS 1988a). Canonical correlations were calculated between the physiological and morphological traits (SAS 1988b). Variance components were calculated for each trait for the model of branches within trees within sites, with leaves as the units, using restricted maximum likelihood methods (SAS 1988b). The variance components are those parts of the total variation which can be attributed uniquely to each stratum in a designed experiment.

When doing simultaneous comparisons of multiple data as in this case, some of the variables tested will by chance indicate a significant correlation. In hindsight a stronger than normal criterion for determining levels of significance would have been necessary. Further statistical analysis was decided against in this case because none of the correlations were significant anyway.

To determine whether there were any significant differences among the populations, PROC nested in SAS (SAS I988) was used to undertake a nested ANOVA on site and tree within site.

Results and Discussion

The results for both Pearson's and the canonical correlations were inconclusive. Consequently, this chapter still highlights the very interesting question: is the success of *B. marginata* partly linked to its intra-population morphological variation? Furthermore, can the apparent intra-population morphological variation in this species be positively correlated with physiological variation?

Although there was no correlation between the morphological and physiological variation of the *B. marginata* populations studied, to have confidence in these results it would be necessary to examine many more populations.

Indeed, the results suggest that the intra-population morphological differences observed at each site were not significant ($P<0.05$) even though there were obvious visual differences in leaf size and shape. Thus, on the basis of field observations, it is hard to be confident that the observed variations in intra-population leaf morphology at each site were not significant.

The results once again highlight the drought and frost resistant nature of *B. marginata*. For example, it took the *RWC* to drop between 28-39% and the frost temperature to fall to between -8.102°C and -12.3°C before 50% damage occurred in the tissues. These results, like the results for ε observed in Chapter 5, indicate the capacity for the maintenance of good cell membrane stability by *B. marginata* during drought and frost events.

Some plant species possess desiccation tolerant protoplasm (Bewley 1974, Levitt 1980 and Gaff 1980). It has been suggested by Bewley (1979), Gaff (1980), Levitt (1980) and Kramer (1983) that desiccation tolerance is primarily due to properties of the protoplasm. Desiccation tolerant plants however, are not exempt from perturbations to their metabolic and cellular integrity but rather these plants are able to limit the degree of desiccation damage to a level that is quickly reparable upon rehydration (Bewley 1979).

The degree to which plant species can tolerate water deficit varies, e.g. according to Bewley and Krochjove (1982) and Levitt (1972) most higher plants are killed at a water loss between 40-90% of their normal water content. Bewley and Krochjove (1982) maintain that provided the attainment of moisture equilibrium with the environment can be prevented, there is no limit to the level of water stress a plant can survive. The reasons for differences in the desiccation tolerance of species are thought to be related to the properties of the protoplasm and protoplasmic membranes (Kramer 1983). In the case of *B. marginata,* it is reasonable to suggest that the excellent desiccation tolerance of this species is a function of its capacity to lower its ε value in response to drought stress, as was observed in Chapter 5. By contrast, plants with desiccation sensitive protoplasm may undergo plasmolysis in response to drought. During plasmolysis, the cell wall is pulled inward with its cell contents. As a result, folds and concavities arise, opposite sides meet (Levitt 1980) and electrolytes leak out from the tissues due to mechanical damage.

Analysis of variance (see Appendix 4) indicates significant differences ($P < 0.05$) among the sites for frost tolerance. Figure 9.1 demonstrates that the most frost tolerant population was from Southarm at sea level with a *T50* of -12.31°C.

The frost results for this experiment are unlike the results for the cline experiment (see Chapter 7) in that the population with the greatest frost tolerance is not from the highest altitude. The Southarm sea level population had the greatest frost tolerance rather than the Ridgeway population at 280 m asl. In the cline experiment however, the Ridgeway population was more frost tolerant than all of the lower altitude populations, including the higher altitude population from Mount Nelson (420 m asl).

It is also of note that unlike the highest population from Mount Wellington in the cline, the highest population from Ridgeway for this experiment was the least desiccation tolerant. It is likely that the altitude effect of the Rideway site (280 m asl only) especially over the time the experiment was conducted, was not great enough to have a significant effect on improving the frost and desiccation tolerances of the *B. marginata* populations.

The mean frost tolerances of the two Ridgeway populations over late November and early December 1994 are very similar to 1993 values obtained in the cline experiment. In this experiment Ridgeway site 1 had a *T50* of -8.975°C and Ridgeway site 2, a *T50* of -8.510°C compared to the cline experiment where the Ridgeway population had a *T50* of -9.309°C and -10.036°C in November and December 1993 respectively. This result shows a certain degree of consistency with regard to the Ridgeway *B. marginata* population exhibiting good levels of frost tolerance outside of the cooler winter months.

The results for the frost experiment again highlight interpopulation physiological variation within *B. marginata*. The sites of the highest and lowest frost tolerance in this instance were both at sea level, so that the significant interpopulation differences cannot be attributed to differences in hardening due to differences in altitude. In addition, the experiments were performed in the summer when cold hardening should have been minimal (see Table 9.1 for temperature details). However, as has been demonstrated in previous chapters, certain species will improve their frost tolerance when exposed to drought stress.

As indicated earlier, the most frost tolerant population is from Southarm. It is also interesting that this population has the greatest level of desiccation tolerance. The type of response exhibited here was also exhibited by *B. marginata* from Mount Wellington in Chapter 7 in which this population overall had the greatest level of frost and desiccation tolerance. In Chapter 7 the superior desiccation tolerance of the Mount Wellington population is measured by its overall lower ε value and its higher R^*a values compared to the other populations. The response in this chapter adds further strength to the observation that useful levels of frost tolerance in *B. marginata* are not confined to the cooler winter months and also occur during the warmer months of the year, probably as an indirect response to hardening to water stress. In the Tasmanian situation, where out of season frosts are not uncommon, the capacity of *B. marginata* to have good levels of frost tolerance all year round would assist this species to survive across a wide range of altitudes in Tasmania. It would be interesting to conduct an experiment to compare the frost and desiccation tolerance of well watered *B. marginata* to *B. marginata* over a period of summer drought.

In the case of the desiccation experiment, the Ψ values indicate that the plants examined at each site were not under much water stress pre-dawn (Table 9.2), because the values of the *Banksia* Ψ were never very negative. For example, the closer the Ψ of a plant is to 0, the less water stress the plant is under. The rationale of performing pre-dawn Ψ is that this is the time of the day when the plant is not transpiring and has had maximum time (overnight) to rehydrate before sunrise. In this chapter, the diurnal changes in *B. marginata* Ψ were not monitored, however, for comparison Dodd and Bell (1993a) examined diurnal changes in *Banksia attenuata* and *B. menziesii.* They recorded that these species exhibited "relatively high" plant Ψ values at dawn of ~-0.2 MPa.

Below are examples of the Ψ values of other species recorded at their least and most stressed times of the day. These examples indicate that the pre-dawn Ψ of the *Banksia* examined were indeed not very negative when compared to the day time Ψ that other species have been recorded at. Lo Gullo and Salleo (1988) looked at diurnal changes in the Ψ of *Olea oleaster* in summer in Sicily at the beginning and end of a drought period: e.g. at 0700 the Ψ in May was -0.25 MPa compared to -1.8 MPa in September. Robichaux (1984) examined diurnal changes in Hawaii of the Ψ of *Dubatia ciliolata* and *D. scabra.* The Ψ of *D. ciliolata* at ~0600 hours was -0.3 MPa compared to -1.2 MPa at ~1400 hours. The Ψ of *D. scabra* at ~0600 hours was -0.1 MPa compared to -7.8 Mpa at ~1400 hours. Losch *et al.* (1982) examined diurnal changes in the Ψ of *Quercus coccifera* in the summer months of July and August 1987 and noted that at 0700 in July the Ψ was -0.8 MPa and in August at 1100 hours the Ψ was -2.5 MPa. As the above examples illustrate, pre dawn Ψ values are usually much less negative the values obtained after sunrise. It would be expected that a plant under severe water stress would have greater difficulty "re-hydrating" overnight than a plant under no water stress, consequently, the pre-dawn Ψ of a drought stresssed plant would be much more negative than the unstressed specimen. Based on the results obtained in this chapter and the results obtained in Chapter 5 (i.e. where the desiccation tolerance of *B. marginata* increased in response to drought stress by the PEG) it is likely that the desiccation and frost tolerance of *B. marginata* could be further improved with greater drought stress.

There was no significant difference ($P > 0.05$) among the populations for the desiccation treatment (see Appendix 4 and Fig. 9.2).

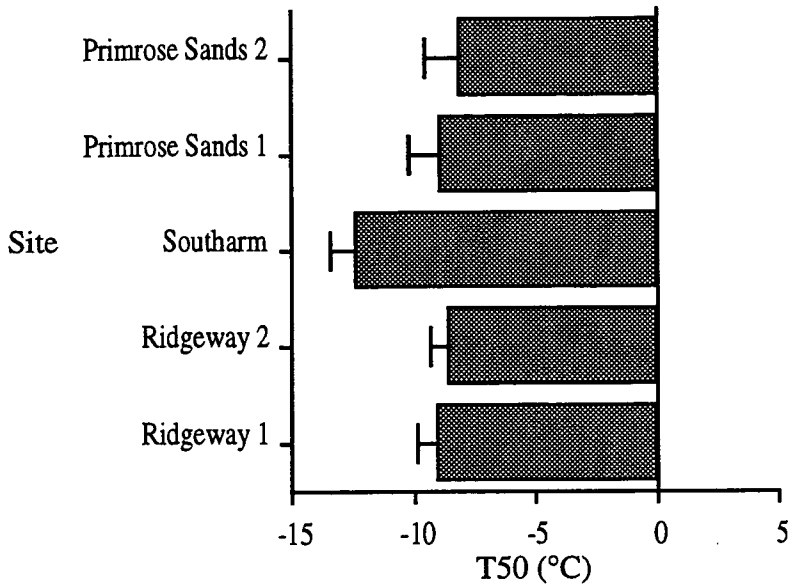

Figure 9.1. Comparative frost tolerance (°C at 50 % tissue damage) of *Banksia marginata.* Standard error bars are shown.

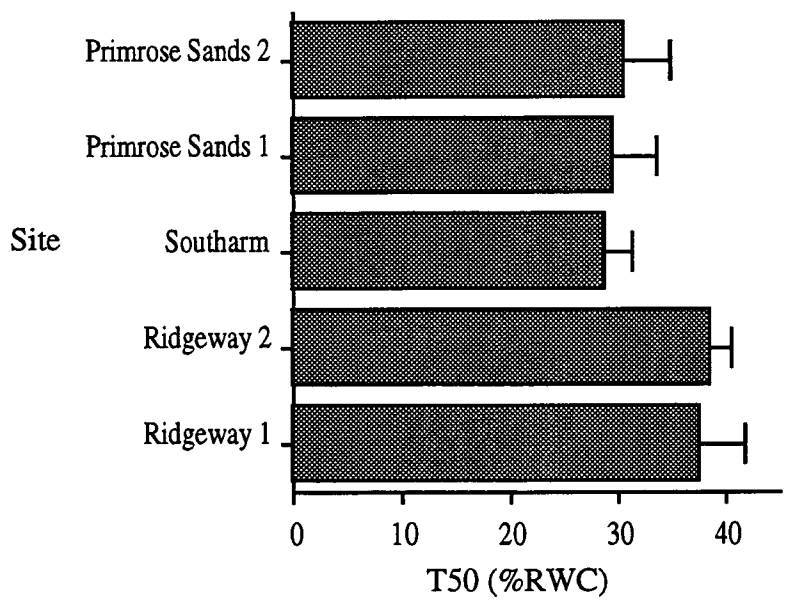

Figure 9.2. Comparative desiccation tolerance (relative water content at 50% tissue damage) of *Banksia marginata.* Standard error bars are shown.

CHAPTER 10 GENERAL DISCUSSION

The significance of the physiology results

This study has been concerned with the history of the genus *Banksia* in Tasmania during the Pleistocene. However, quite apart from the attempt to account for the Pleistocene extinction of *B. kingii* and *B. strahanensis* from Tasmania, the physiology results provide insight into how certain species of *Banksia* respond to climatic stress.

Plants use many mechanisims to resist frost and drought damage. The results highlight the significance of bulk elastic modulus and apoplastic water content for tolerance of drought and cold stress in *Banksia*. All of the *Banksia* species examined underwent reductions in ε in response to drought stress. Increased cell wall elasticity facilitates the maintenance of cell turgor to lower relative water contents than would otherwise occur (Andersen and McLaughlin 1991). *Banksia marginata* was also able to increase its apoplastic water content in response to both drought and cold stress. Indeed, the apparent inherent greater stress tolerance of *B. marginata* was shown by its ability to adjust elasticity and apoplasm water. High apoplastic water contents have been observed in plants pre-conditioned to water stress (Blake and Tschaplinksi 1992). The flow of water into the apoplasm has been reported to protect living cells from damage by protecting them from sudden water loss through subsequent dehydration (Blake and Tschaplinksi 1992). Indeed, it was apparent from the results for the hydroponic drought stress experiments (i.e. bulk elastic modulus and apoplastic water content) that *B. marginata* had extremely desiccation tolerant tissues. This was confirmed when the desiccation tolerance of *B. marginata* was examined in Chapter 9 (i.e. 50% tissue damage occurred at relative water contents between 28-39%). It is however, important to keep in mind that this is only an estimate. Furthermore according to Kummerow (1973) the compact palisade parenchyma and the generally well developed sclerenchymatic tissue of *Banksia* provide the leaves with a certain degree of protection against irreparable damage from severe drought stress (Kummerow 1973). The results from the desiccation experiments support these observations.

Numerous records of the significance of osmotic adjustment to plant survival of drought and cold have been documented (e.g. Abrams 1988, Valentini *et al.* 1990, Rhizopoulous and Mitrakos 1990). However, in this study it is apparent that the contribution of osmotic potential to the survival of drought stressed *Banksia* was of little importance. It is significant that all of the populations of *Banksia* (with the exception of *B. saxicola* from Wilsons Promontory and *B. spinulosa* var. *cunninghamii*) underwent a significant decrease in osmotic potential in response to the drought stress treatment imposed through the hydroponic system. Indeed, the results for osmotic potential are one of few known reports of what has been termed osmotic de-adjustment (e.g. Blake and Tschaplinksi 1992, Gebre *et al.* 1994). Blake and Tschaplinski (1992) have observed osmotic de-adjustment in plants sensitive to drought stress, however the *Banksia* species examined were not sensitive to drought stress. The *Banksia* species appeared to compensate for their incapacity to undergo osmotic adjustment by being able to increase their cell wall elasticity and apoplastic water contents in response to drought stress.

Furthermore, the results are important in that they provide an example of the similarity between the physiological mechanisms behind the drought and frost tolerance of certain species of *Banksia*. For example, for the cline study, it is significant that of the *B. marginata* populations studied, the most frost tolerant population (i.e. Mount Wellington) generally had the least negative osmotic potential, at the same time as having the highest cell wall elasticity and apoplastic water contents. Similar to the drought stress results described above, these frost results imply that *B. marginata* does not depend on osmotic adjustment to survive climatic stress. This result is contrary to the findings of O'Neil (1983), Doi *et al.* (1986), and Valentini *et al.* (1990) who have found a negative correlation between osmotic potential and frost tolerance in plants.

Further similarities in the physiological mechanisms used by some plants to survive drought and cold stress are displayed by the apoplasm. It is apparent that whether *B. marginata* is exposed to hardening by drought or cold, either stimulus will result in an improved tolerance to both of these stresses. For example, high apoplastic water contents have been observed in both freezing tolerant plants (Larcher 1980) and plants which have been pre-conditioned to water stress (Blake and Tschaplinksi 1994). As indicated above, in relation to drought, the flow of water into the apoplasm has been

reported to protect cells from damage by protecting them from sudden water loss during subsequent dehydration stress (Blake and Tschaplinksi 1994). In a similar way, freezing tolerant plants avoid intracellular ice crystal formation through the displacement of symplastic water to the apoplast (Larcher 1980). In either case, the lowering of the symplastic water content to supplement the apoplastic water content would result in some degree of intracellular dehydration. This could explain the extremely desiccation tolerant tissues of *B. marginata* and why the species responds to drought stress by increasing its cell wall elasticity.

Extinction of Banksia kingii *and* B. strahanensis *from Tasmania*

The fossil record implies that *B. strahanensis* and *B. kingii* were present in Tasmania during the Pleistocene. Many plant species are vulnerable to extinction as a consequence of climatic change (Knoll 1984, Stanley 1987) particularly climatic cooling and glaciations (Leopold 1967).

An attempt was made to highlight any physiological weakness in the drought and cold tolerance of the closest living relatives of the fossil species *B. strahanensis* and *B. kingii*, which may have contributed to their Pleistocene extinction from Tasmania.

Overall, the physiology results provide little insight into the reasons behind the extinction of the *Banksia* species from Tasmania during the Pleistocene. The results imply that the extinction of *B. strahanensis* and *B. kingii* from Tasmania cannot necessarily be linked to a weakness in their physiological tolerance to drought and cold stress.

The fossil evidence indicates that *B. kingii*, unlike *B. strahanensis*, managed to survive the rapid changes of climate and landform during the middle Pleistocene, to become extinct from Tasmania in the latter part of this period (Jordan 1992).

The populations of the closest living relatives of *B. kingii* which most resemble this species (i.e. *B. saxicola* and *B. canei*) occur today in exposed, moderately wet, high altitude areas (1200 m asl) in the Grampians of Victoria and the top of the Kybean Range in southern New South Wales respectively (Jordan 1992). Based on the morphology of the closest living relatives from

these high altitude populations, Jordan (1992) suggests that *B. kingii* was probably adapted to harsh, cold, but not particularly dry conditions.

The water relations results however, do not entirely support the above theory. Indeed, seedlings of *B. canei* and *B. saxicola* from Mount William were able to significantly decrease their ε values in response to the drought treatment. However, the lower altitude population of *B. saxicola* from Wilsons Promontory was unable to significantly decrease ε and neither *B. canei* nor *B. saxicola* were able to significantly increase their apoplastic water contents in response to drought.

Seedlings of *B. saxicola* from Mount William and Wilsons Promontory were particularly sensitive to the out of season frost treatment. However, it is unlikely that out of season frosts would have significantly impacted on the population size of *B. kingii* during the dry Last Glacial in view of the capacity of the closest living relatives of this species to greatly improve their frost tolerance in response to drought stress.

Banksia spinulosa var. *cunninghamii* is more widely distributed throughout mainland Australia than the closest living relatives of *B. kingii*. Based on its current distribution and the physiology results, it is more likely that the distribution of this species was more limited by cold stress than was *B. kingii*. For example, *B. spinulosa* var. *cunninghamii* was overall the least frost tolerant of the species examined. Based on this, it is reasonable to suggest that the earlier extinction of *B. strahanensis* than *B. kingii* may have resulted from its greater sensitivity to the cold stress of the Pleistocene. Perhaps *B. strahanensis* became extinct from Tasmania during the Last Glacial, having retracted to refugia in Victoria and New South Wales from which it has since been able to radiate. It is interesting however, that the morphological features of *B. spinulosa* var. *cunninghamii* (i.e. narrow, sclerophyllous leaves with revolute margins) are characteristic of plants from cold, dry or oligotrophic conditions (Jordan 1992).

The poorer frost tolerance displayed by *B. spinulosa* var. *cunninghamii* cannot be attributed to the fact that the population examined was grown from seed collected from the coast. For example, the frost tolerance of seedlings of *B. saxicola* from Wilsons Promontory (grown from seed collected nearby to *B. spinulosa* var. *cunninghamii*), was greater for two thirds of the experiments

(though not always significantly so). Furthermore, coastal populations of *B. marginata* were not necessarily less frost tolerant than the inland populations of this species. Eiga and Sakai (1984) found that coastal populations of the eastern Pacific *Abies sachalinensis* var. *nemureensis* were more frost tolerant than inland populations, even though the coastal habitat is more mild than that experienced by the inland populations in winter.

The seedlings of *Banksia spinulosa* var. *cunninghamii* were able to significantly decrease both ε and increase R^*a in response to the drought stress treatment where as *B. canei* and *B. saxicola* were only able to decrease their ε. This result suggests that *B. strahanensis* was better equipped to deal with drought stress than *B. kingii*, even though Jordan (1992) indicates that *B. strahanensis* probably did not persist for as long into the Pleistocene. It is interesting however, that *B. spinulosa* var. *cunninghamii* was more drought tolerant than *B. canei* and *B. saxicola*, however, unlike *B. canei* and *B. saxicola* its frost tolerance did not substantially increase with drought stress.

If *B. kingii* (in the form of *B. saxicola* or *B. canei*) became extinct from Tasmania during the Last Glacial of the Pleistocene, it is worth noting that *B. saxicola* and *B. canei* have not been able to significantly expand their range during the current interglacial. This may be due to a poor capacity to compete with other species for resources. The bottle-necking of *B. saxicola* into two refugia may have led to the significant erosion of the species gene pool to a point where the species is unable to compete with other species beyond the geographical range of its current distribution. Its niche requirements may have become too specialised. Indeed, according to Salkin and Hallam (1978), the four distinct forms of *B. canei* are likely to have resulted from a blockage in inter-population gene flow due to their geographical isolation from one another.

The absence of *B. spinulosa* var. *cunninghamii*, *B. saxicola* and *B. canei* from Tasmania today may be explained by their inability to disperse into Tasmania after the submergence of the land bridge which once connected Wilsons Promontory and Tasmania during the Last Glacial. Island taxa are rarely thought to re-invade continents (Darlington 1968). Land bridges serve plants in two ways:

(1). they are a connection between otherwise inaccessible places and,
(2). they are refuges for groups of plants during periods of extensive glaciation.

The question however, still remains 'How and when did *B. strahanensis* and *B. kingii* get to Tasmania in the first place?' According to Darlington (1968) most movements of plants and animals have been from continents to islands. Indeed, if *B. strahanensis* and *B. kingii* were sensitive to the cold and drought stress of the Last Glacial, how could they have migrated across the land bridge toward Tasmania, since the only time the bridge was exposed, was during glacial events when the sea level was ~140 m lower? With this consideration in mind, unless *B. kingii* and *B. strahanensis* arrived in Tasmania by another route, it is more likely these species were sensitive to the warmer conditions of the interglacial than the cool, dry conditions of the Last Glacial (see discussion later on).

Although the drought and cold stress tolerance of the closest living relatives of the fossil species were not often significantly different from that displayed by the different populations of Tasmanian *B. marginata*, it must be questioned whether the level of stress tolerance that each of the closest living relatives displayed would have been sufficient to enable the fossil species to survive the glacial climates of the Pleistocene. As indicated previously, in the Snowy Mountains of New South Wales, temperatures were between 8-10° C cooler at the height of the Last Glacial than at present (Costin 1972). With this scale of temperature difference, it is very likely that the population numbers of all the *Banksia* species examined, would have been reduced to some degree by the glacial cold and drought stress.

Finally, it cannot be totally discounted that perhaps the physiology results only reflect the more recent adaptation of the populations of each of the species examined to their current habitats. The results may not accurately reflect the physiological response these species would have displayed under the climatic stress of the Pleistocene. However, as has been pointed out earlier in this thesis, plant stress physiology does not always reflect species' adaptation to their to current habitats.

Alternative Hypotheses

The results have stimulated the creation of new hypotheses regarding *Banksia* extinction from Tasmania and have resulted in re-newed interest in hypotheses previously formulated by Jordan (1992). The hypotheses, outlined below, will need to be investigated if the extinction of *Banksia* species from Tasmania during the Pleistocene is to be better understood.

Interglacial extinction

As has already been suggested, it is possible that the extinction of *B. kingii* and *B. strahanensis* occurred during the interglacial period of the Pleistocene. Rapid warming occurred at the end of the glacials, with most of the range from maximum cold to maximum warmth occurring in less than 10 000 years (Jordan 1992). By contrast, the cooling phase between the interglacial and glacial periods was slower, over periods of tens of thousands of years (Bartlein and Prentice 1989). Since most of the Pleistocene was cold, the comparatively rapid entry into the warmer conditions of the interglacials may have placed considerable stress on the fossil *Banksia* species (Jordan 1992).

According to Knoll (1984), whether climatic change results in high levels of extinction or not, is probably dependent not so much on the absolute values of mean annual temperature or precipitation changes but on the capacity of a species to migrate to suitable areas of refuge.

In Tasmania, there is very little land mass to the south to which plant species could have migrated, to escape the warmer conditions of the interglacial (Jordan 1992). Consequently, options for survival in cool refugia would have been limited to the migration of species to higher altitudes (Jordan 1992). It is however, important to question why the *Banksia* species would have successfully survived in cool mainland refugia during the Last interglacial of the Pleistocene when by virtue of their latitude, the Victorian refugia would very likely have been warmer than Tasmanian refugia of equivalent altitude. Perhaps then, the extinction of *B. kingii* and *B. strahanensis* from Tasmania is more related to their failure to be competitive for resources during the interglacial of the Pleistocene.

Disease

Disease may have caused the extinction of *Banksia kingii* and *B. strahanensis* from Tasmania during the Pleistocene. There is, however, evidence to suggest that epidemics, parasitism and disease have played little more than a minor role in species extinction (Edwards 1967). However, when physical stress such as drought and cold (common during a glacial event) is combined with disease and/or predation, it is possible that populations of certain species could be weakened to the point of extinction. This could only occur if stress was particularly severe and/or if the population size of a species was small and with a gene pool of limited diversity.

A study on *Eucalyptus viminalis* in the Tasmanian Midlands by Neyland (1996) has revealed that although drought stress has played a major role in the decline of this species, it is the effect of predation by possums and insects on plants in an already stressed and weakened state of drought that has led to widespread, massive regional tree death in the Midlands of Tasmania.

Phytophthora cinnamomi is a present day example of a pathogen to which *Banksia* species are vulnerable. *Phytophthora cinnamomi* is a soil borne pathogenic fungus. This disease attacks the root system of woody plants and is responsible for Jarrah dieback in Western Australia. The fungus is distributed world wide and was introduced to Australia at least 40 years ago (Podger *et al.* 1990). Today, populations of this fungus species are scattered throughout much of Tasmania in areas below 800 m asl and which have a mean annual rainfall of at least 600 mm, affecting about 136 species in Tasmania (Podger *et al.* 1990) . Although there is no fossil evidence to suggest that this fungus was present in Tasmania during the Pleistocene (G.J. Jordan pers. comm.) it is an example of a pathogen which seriously affects the health of many native Australian species. Under the different climatic conditions of the previous glaciations, other pathogens would have been in existence, which combined with the stress of drought and cold may have been locally damaging to populations of *B. kingii* and *B. strahanensis*.

The short life cycles and rapid breeding time of many pathogens is conducive to mutation. Indeed, in Tasmania recently, a new form of *Phytophthora* has

been isolated from an altitude higher than the upper limits that *P. cinnamomi* has been previously recorded in Tasmania (J. Winham pers. comm.).

Fire

Eradication of *B. kingii* and *B. strahanensis* by fire must also be considered, although in general, extant *Banksia* species are adapted to fire.

The sensitivity of the closest living relative of *B. kingii* (*B. canei*) to fire is reputedly unknown, but George (1984) and Jordan and Hill (1991) suggest it is fire sensitive. However, there are some unopened seed follicles in the fossil infructescence of *B. kingii* which suggests an adaptation to fire (Jordan and Hill 1991). According to George (1984) and Jordan and Hill (1991) the retention of seed in unopened follicles is a common adaptation to fire. It is reasonable to suggest that *B. kingii* was adapted to release seed post fire.

Banksia spinulosa var. *cunninghamii* (closely related to *B. strahanensis*) and *B. saxicola* (related to *B. kingii*) are generally killed by fire and regenerate from seed released from cones in response to fire (George 1984).

The response of *B. marginata* to fire is varied. Some populations survive fire by lignotubers, epicormic buds or suckers. Other populations are killed and rely on regeneration from seed (George 1984). Where the other *Banksia* species are generally killed by fire and rely on regeneration exclusively by seed, *B. marginata* does not.

Too frequent fires can wipe out populations, by stopping individuals within them from reaching reproductive maturity. Where the population numbers of a species are small, extinction from too frequent fire is a distinct possibility. Where a plant species is capable of vegetative regeneration after fire (e.g. *B. marginata*), species extinction is less likely to occur from too high fire frequencies, especially if the species is widely dispersed as *B. marginata* may have been.

Fossil record

Current populations of *B. marginata* are well recognised for their striking morphological variation. Therefore, it cannot be totally discounted that the macrofossils identified by Jordan and Hill (1991) as closely related to *B. saxicola*, *B. canei* and *B. spinulosa* var. *cunninghamii* are just other forms of *B. marginata*. Jordan (pers. comm.) however, is completely confident that there is no risk that *B. strahanensis* (closest living relative *B. spinulosa* var. *cunninghami*) could be a form of *B. marginata*. He concedes that there is a very slight possibility that *B. kingii* (closest living relative of *B. canei* and *B. saxicola*) could be *B. marginata*. Only the discovery of further macrofossil deposits could alleviate this doubt.

Loss of pollinator or ecologically essential species

Jordan (1992) suggests that the extinction of many of the Tasmanian *Banksia* species may have resulted from the loss of certain species which were ecologically essential to their existence. It is unlikely the extinction of Pleistocene *B. kingii* and *B. strahanensis* was associated with the loss of their pollinators since information drawn from Trounson and Trounson (1989) indicates no lack in Tasmania of bird and insect species responsible for the pollination of the extant closest living relatives in New South Wales and Victoria. In addition, according to Jordan (1992) these species all produce seed in cultivation in Tasmania. It is possible however, that had the pollinators of *B. kingii* and *B. strahanensis* been eliminated from Tasmania during the Pleistocene, their presence in the State today, may be the result of recent migration back.

Competition

During the glacial events of the Pleistocene, conditions were much cooler and drier than at present (Macphail 1978, Sigleo and Colhoun 1981, Bowden 1983), thus it is likely that water was a resource often in short supply, particularly on the east coast of Tasmania, hence the likelihood of competition for this resource. Obviously, species with narrow distributions

and low genetic diversity would have been less likely to survive prolonged periods of competition in response to climatic stress than species with wider distributions and greater genetic diversity.

Synchronicity of plant growth with season

Banksia species tend to begin growth in late spring-early summer, continuing growth well into summer. It appears mean daily temperature has to rise above 16-18°C before growth will begin, a threshold more characteristic of tropical species (Fitzpatrick and Nix 1970). Growth continues spasmodically through summer whenever soil water is available (Holmes 1960, Martin and Specht 1962). Had the *Banksia* species been poorly adapted to the glacial conditions they may have been unable to synchronise plant growth with the rhythmicity of climate. This would have restricted their range, perhaps leading to species extinction.

About the success of *Banksia marginata*

This study has also sought to address the question " How did *B. marginata* manage to survive and/or evolve during the Quaternary when all except one other *Banksia* species became extinct from the Tasmanian mainland?"

The origin of *B. marginata* in Tasmania remains unknown. In spite of its current ubiquity and a macrofossil record for the tribe Banksieae in the region (Hill and Christophel 1988) there is no known fossil record for *Banksia marginata* in Tasmania and the Australian mainland. There is a large gap in the Tasmanian fossil record from 25-2.5 Ma (million years before present) (Hill *et al.* 1992), this may partly explain its absence.

Jordan and Hill (1991) however, are almost certain that *B. marginata* was present in Tasmania at the time when the fossilisation of *B. kingii* and *B. strahanensis* occurred. It is probable that *B. marginata* was present in Tasmania at least during the Pleistocene, perhaps having retracted to refugia in response to climatic stress imposed by the glacial/interglacial events of this period. It is possible that fossil beds containing this species have not yet been sampled, or by chance the species has not been fossilised. Alternatively, the lack of fossil evidence for *B. marginata* may indicate that it is recently evolved, with speciation being facilitated by environmental change as hypothesised by Prober (1989). If this is the case however, it is interesting that the species should be so morphologically diverse and widespread on the Australian mainland. There is no doubt however, that Tasmania has had a complex climatic history, with the glacial/interglacial dynamics perhaps providing the stimulus for speciation. Similarly, Costin (1954) has suggested that the abundance of plant species endemic to Kosciusko and the Kybean Ranges is due to the complex geological and climatic histories of these regions providing the stimulus for vigorous speciation. Additionally, *B. marginata* hybridises with other members of the genus (George 1981, Salkin *circa* 1983) thus indicating the potential for rapid evolution. Regardless of the exact history of *B. marginata*, its success at invading and occupying a wide range of habitats today is clear.

When examined, the physiological stress tolerance of *B. marginata* was often no more remarkable than that of the closest living relatives of the fossil

species, i.e. *B. kingii* and *B. strahanensis.* However, *B. marginata*, in addition to *B. spinulosa* var. *cunninghamii* were the only two species capable of increasing their apoplastic water content in response to drought stress. Perhaps this would have given those species a better chance of survival than *B. kingii* should water have been limited during the Last Glacial. It is very unlikely however that this physiological characteristic alone would have enabled it to survive at a time when the other *Banksia* species were unable to.

The success of *B. marginata* is more likely to be related to a diverse gene pool combined with a capacity for physiological and morphological plasticity. Experimental evidence from the plant physiology experiments support this theory.

Banksia marginata seems to have a genetic potential which allows for a high degree of plasticity (Kummerow 1973). However, *B. spinulosa* var. *cunninghamii, B. saxicola* and *B. canei* also have the capacity for physiological plasticity, as evident from the experiments, i.e. being able to harden in response to certain of the stress treatments.

According to Kummerow (1973) the genetic potential for plasticity in *B. marginata* facilitates the production of structures which ensure high photosynthetic efficiency provided that water is available.
More systematic studies are needed to determine why certain species respond to new environments by a plastic response and others by a genetic ecotypic response (Mooney 1980).

The limited isozyme work done i.e. on the two cline populations of *B. marginata* also implies that genetic diversity among the populations of this species may be another key to its success. The isozyme results for the University and Mount Wellington populations supported the results for the cline physiology experiments. These populations were significantly different from one another at a physiological level and the isozyme differences imply that there could indeed be a genetic basis to the physiological differences observed. Those species which possess greater genetic diversity, and/or capacity for phenotypic plasticity than other species will be more successful under stressful climatic conditions than species with more limited genetic variation and capacity for physiological and morphological plasticity.

It is very likely that the size of each *B. marginata* population would have been drastically reduced as the species retracted to refugia in response to the Pleistocene climatic stress. It is through the bottle neck process that speciation of *B. marginata* may have occurred in the refugia. According to Mielke (1989) the glaciation appears to have stimulated speciation. For example, the most highly successful family of plants, the Compositae, has 20 000 species. The family originated in the Oligocene and under went a major evolution during the ice age (Leopold 1967).

To conclude, the success of *B. marginata* in Tasmania today cannot be disputed. The success of this species is likely to be the result of a combination of the following:

- the capacity of the species for physiological plasticity (i.e. populations of this species have been recorded to harden and de-harden in response to climatic stress). Consequently, certain populations of this species have been recorded to tolerate extreme desiccation and freezing stress;

- the capacity of the species to undergo dual hardening in response to either drought or cold stress, i.e. drought stress seems to promote cold tolerance and vice the versa;

- the possession of a diverse gene pool. This is apparent from the significant physiological differences observed among the different populations of this species and the results from the isozyme study. Indeed, diversity of the species gene pool is also hinted at by the vast range of morphological variation displayed by this species. The genetic diversity of *B. marginata* warrants further study.

It is very likely that *B. marginata* survived the climatic stress of the Pleistocene in refugia. During the current interglacial, with genetic diversity to its advantage, this species has managed to radiate out from its refugia to occupy the wide range of niches made available to it through the plant extinctions of the Pleistocene.

The physiology results indicate that the tolerance of *B. marginata* to drought and cold stress was little more than marginally better than that of the closest living relatives of the fossil species. So it is not clear whether the extinction of *B. kingii* and *B. strahanensis* was a consequence of their physiological weakness to Pleistocene drought and cold stress. Rather, it is more likely that *B. kingii* and *B. strahanensis* did not survive the Pleistocene in Tasmania because they did not possess any of the following attributes which Jordan (1992) deemed necessary for plants to survive the climatic changes of the Pleistocene, i.e *B. kingii* and *B. strahanensis* probably did not:

(1). possess a sufficiently wide physiological tolerance to enable survival in refugia,
(2). have sufficiently rapid rate of seed dispersal to enable migration to areas within their range of physiological tolerance and,
(3). have the capacity to evolve quickly enough to adapt to the climatic conditions (Jordan 1992).

It is apparent from this study that the Pleistocene survival of *B. marginata* can be at least partly attributed to its possession of a wide range of physiological tolerance enabling survival in refugia.

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APPENDICES

Note: that the ANOVA were carried out on data in bars (unless otherwise stated) for the osmotic potential, water potential at wilt and bulk elastic modulus - not MPa as in the graphs.

APPENDIX 1

Chapter - 5 Water Relations Experiments

ANOVA Tables for the Hydroponic Drought Stress Experiments

Note: that the ANOVA were carried out on data in units of MPa for osmotic potential, water potential at wilt and bulk elastic modulus.

Note: that Birches Inlet data were accidentally not included in the data anaysis for Chapter 5.. The results for Birches Lnlet however, have been plotted in the graphs of this chapter.

Note: well= Mount Wellington, gard=The Gardens, blow=Blowhole, mela=Melaleuca, snug=Snug, tour=Cape Tourville, hauy=Cape Hauy, saxwp=*B. saxicola* from Wisons Promontory, saxwill=*B. saxicola* from Mount William, spin=*B. spinulosa* var. cunninghamii, and canei=*B. canei.*

Osmotic Potential at Full Turgor

Source	DF	SS	MS	F	P
Species	10	14.65986272	1.46598627	9.68	0.0001
Treatment	1	6.04794002	6.04794002	39.93	0.0001
Species*Tmt	10	1.84547761	0.18454776	1.22	0.2865
Residual	115	17.41734861	0.15145521		
Total	136	39.97062895			

Table 5a

Relative Water Content at Turgor Loss

Source	DF	SS	MS	F	P
Species	10	608.7759302	60.8775930	2.40	0.0124
Treatment	1	555.0217440	555.0217440	21.92	0.0001
Species*Tmt	10	385.5255503	38.5525550	1.52	0.1400
Residual	115	2911.703722	25.319163		
Total	136	4469.278958			

Table 5b

Water Potental at Turgor Loss

Source	DF	SS	MS	F	P
Species (Sp.)	10	25.93668192	2.59366819	3.94	0.0001
Treatment	1	7.84757707	7.84757707	11.92	0.0008
Species*Tmt	10	9.95872205	0.99587220	1.51	0.1437
Residual	115	75.73163692	0.65853597		
Total	136	120.4733930			

Table 5c

Bulk Modulus of Elasticity

Source	DF	SS	MS	F	P
Species (Sp.)	10	222.6562370	22.2656237	4.49	0.001
Treatment	1	284.1498389	284.1498389	57.25	0.001
Species*Tmt	10	66.7085973	6.6708597	1.34	0.2160
Residual	113	560.8895409	4.9636243		
Total	134	1195.466141			

Table 5d

Apoplastic Water Content

Source	DF	SS	MS	F	P
Species (Sp.)	10	0.27373725	0.027373772	1.92	0.0493
Treatment	1	0.33738492	0.33738492	23.68	0.0001
Species*Tmt	10	0.59613836	0.05961384	4.18	0.0001
Residual	112	1.59586252	0.01424877		
Total	133	2.68504088			

Table 5e

CONTROLS

ANOVA Tables for one way analysis

Osmotic Potential at Full Turgor

Source	DF	SS	MS	F	P
Species	10	5.58674266	0.55867427	6.18	0.0001
Residual	68	6.14978284	0.09043798		
Total	78	11.73652550			

Table 5.1a

%Relative Water Content at Turgor Loss

Source	DF	SS	MS	F	P
Species	10	261.632406	26.1632406	1.59	0.1291
Residual	68	1120.470714	16.4775105		
Total	78	1382.103120			

Table 5.1b

Water Potential at Turgor Loss

Source	DF	SS	MS	F	P
Species	10	57.41791301	1.94454045	2.30	0.0214
Residual	68	19.44540449	0.84438107		
Total	78	76.86331749			

Table 5.1c

Bulk Modulus of Elasticity

Source	DF	SS	MS	F	P
Species	10	164.430396	16.4430397	3.27	0.0018
Residual	66	332.0726092	5.0314032		
Total	76	496.5030058			

Table 5.1d

Apoplastic Water Content

Source	DF	SS	MS	F	P
Species	10	0.50233596	0.05023360	4.11	0.0002
Residual	67	0.81830928	0.01221357		
Total	77	1.32064524			

Table 5.1e

DROUGHT TREATMENT

Osmotic Potential at full Turgor

Source	DF	SS	MS	F	P
Species	10	9.05818487	0.90581849	3.78	0.0009
Residual	47	11.2675657	0.23973544		
Total	57	20.3257506			

Table 5.2a

%Relative Water Potential at Turgor Loss

Source	DF	SS	MS	F	P
Month	10	666.5258179	66.6525818	1.75	0.0975
Residual	47	1791.233006	38.1113406		
Total	57	2457.758824			

Table 5.2b

Water Potential at Turgor Loss

Source	DF	SS	MS	F	P
Month	10	15.70762208	1.57076221	4.03	0.0005
Residual	47	18.31372392	0.38965370		
Total	57	34.02134600			

Table 5.2c

Bulk Modulusof Elasticity

Source	DF	SS	MS	F	P
Month	10	132.7614391	13.2761439	2.73	0.0099
Residual	47	228.8169317	4.8684454		
Total	57	361.5783708			

Table 5.2d

Apoplastic Water Content

Source	DF	SS	MS	F	P
Month	10	0.34859078	0.03485908	2.02	0.0537
Residual	45	0.77755324	0.01727896		
Total	55	1.12614402			

Table 5.2e

Tukey's Test

Osmotic Potential at Full Turgor (MPa)

Tukey Grouping	Mean	N	SPECIES
A	-1.2683	4	well
A			
A	-1.2904	5	gard
A			
B A	-1.4303	5	blow
B A			
B A	-1.4347	5	mela
B A			
B A	-1.4435	5	snug
B A			
B A C	-1.6021	7	saxwp
B A C			
B A C	-1.7905	10	tour
B A C			
B A C	-1.7994	9	canei
B A C			
B A C	-1.8000	10	hauy
B C			
B C	-1.9052	8	saxwill
C			
C	-2.1570	11	spin

Figure 5.1a

%Relative Water Content at Turgor Loss

Tukey Grouping	Mean	N	SPECIES
A	82.294	7	saxwp
A			
A	81.100	5	snug
A			
A	80.764	11	spin
A			
A	80.675	8	saxwill
A			
A	80.050	5	mela
A			
A	78.750	10	hauy
A			
A	78.411	9	canei
A			
A	77.825	4	well
A			
A	77.350	5	gard
A			

A		77.020	10	tour
A				
A		76.075	5	blow

Figure 5.2a

Water Potential at Turgor Loss (MPa)

Tukey Grouping		Mean	N	SPECIES
A		-1.6650	4	well
A				
A		-1.7620	5	gard
A				
B	A	-1.8126	5	mela
B	A			
B	A	-1.9000	5	blow
B	A			
B	A	-2.1571	7	saxwp
B	A			
B	A	-2.5750	10	tour
B	A			
B	A	-2.5813	8	saxwill
B	A			
B	A	-2.7225	10	hauy
B	A			
B	A	-2.7556	9	canei
B	A			
B	A	-3.0045	11	spin
B				
B		-3.4960	5	snug

Figure 5.3a

Bulk Elastic Modulus (MPa)

Tukey Grouping		Mean	N	SPECIES
A		8.776	10	spin
A				
B	A	7.103	8	saxwill
B	A			
B	A	6.533	10	tour
B	A			
B	A	5.712	9	canei
B	A			
B	A	5.468	5	mela
B	A			
B	A	5.427	10	hauy
B	A			
B	A	5.390	4	snug
B	A			
B	A	5.094	7	saxwp
B				
B		4.405	5	well
B				
B		3.708	5	blow
B				
B		3.704	5	gard

Figure 5.4a

Apoplastic Water Content

Tukey Grouping	Mean	N	SPECIES
A	0.36968	7	saxwp
A			
A	0.36073	9	canei
A			
B A	0.29377	9	hauy
B A			
B A C	0.27829	10	tour
B A C			
B A C	0.27237	8	saxwill
B A C			
B A C	0.26069	5	snug
B A C			
B A C	0.25934	11	spin
B A C			
B A C	0.17427	5	gard
B C			
B C	0.15088	5	blow
B C			
B C	0.13304	5	mela
C			
C	0.07615	4	well

Figure 5.5a

Drought Stress Treatment

Osmotic Potential at Full Turgor (MPa)

Tukey Grouping	Mean	N	SPECIES
A	-0.6333	4	well
A			
A	-0.8904	5	blow
A			
A	-0.8935	5	mela
A			
A	-0.9001	4	snug
A			
A	-1.0528	5	canei
A			
A	-1.0651	5	gard
A			
B A	-1.1831	6	tour
B A			
B A	-1.2922	4	saxwill
B A			
B A	-1.4222	8	hauy
B A			
B A	-1.5053	6	saxwp
B			
B	-2.1414	6	spin

Figure 5.1b

%Relative Water Content at Turgor Loss

Tukey Grouping	Mean	N	SPECIES
A	81.017	6	saxwp
A			
A	78.833	4	well
A			
A	77.133	4	snug
A			
A	77.076	5	mela
A			
A	76.883	6	tour
A			
A	74.313	8	hauy
A			
A	73.050	5	blow
A			
A	72.576	5	gard
A			
A	72.565	4	saxwill
A			
A	71.050	6	spin
A			
A	69.325	5	canei

Figure 5.2b

Water Potential at Turgor Loss (MPa)

Tukey Grouping		Mean	N	SPECIES
A		-1.0833	4	well
A				
A		-1.3745	4	snug
A				
A		-1.4250	5	mela
A				
A		-1.7380	5	gard
A				
A		-1.7500	5	canei
A				
A		-1.8260	5	blow
B	A			
B	A	-2.0333	6	tour
B	A			
B	A	-2.0500	5	saxwill
B	A			
B	A	-2.1750	6	saxwp
B	A			
B	A	-2.3563	8	hauy
B				
B		-3.0917	6	spin

Figure 5.3b

Bulk Modulus of Elasticity (MPa)

Tukey Grouping	Mean	N	SPECIES
A	5.550	6	spin

A				
A		4.818	6	saxwp
A				
A		3.570	8	hauy
A				
A		2.907	5	saxwill
A				
A		2.379	5	gard
A				
A		2.088	6	tour
A				
A		1.943	5	mela
A				
A	B	1.744	5	blow
A	B			
A	B	1.031	4	snug
A	B			
B		0.911	5	canei
B				
B		0.861	4	well

Figure 5.4b

Apoplastic Water Conent

Tukey Grouping	Mean	N	SPECIES
A	0.45539	4	well
A			
A	0.44721	4	snug
A			
A	0.44409	6	tour
A			
A	0.42627	5	blow
A			
A	0.33995	5	mela
A			
A	0.32015	6	saxwp
A			
A	0.31366	8	hauy
A			
A	0.29574	5	gard
A			
A	0.29340	5	bi
A			
A	0.28401	4	canei
A			
A	0.28182	5	spin
A			
A	0.18108	4	saxwill

Figure 5.5b

APPENDIX 2

Chapter 6 - Frost Tolerance Experiments ANOVA Tables

Note: well= Mount Wellington, gard=The Gardens, blow=Blowhole, mela=Melaleuca, snug=Snug, tour=Cape Tourville, hauy=Cape Hauy, birches=Birches Inlet, saxwp=*B. saxicola* from Wisons Promontory, saxwill=*B. saxicola* from Mount William, spin=*B. spinulosa* var. cunninghamii, and canei=*B. canei.*

PEG stressed only

Source	DF	SS	MS	F	P
Species	11	1129.244128	102.658557	7.56	0.0001
Residual	48	652.040015	13.584167		
Total	59	1781.284143			

Table 6a

Tukey's Test

Tukey Grouping		Mean	N	SPECIES
A		-9.099	5	spin
A				
A		-9.731	5	saxwp
A				
B	A	-10.375	5	tour
B	A			
B	A	-11.335	5	mela
B	A			
B	A	-11.336	5	hauy
B	A			
B	A	-11.489	5	gard
B	A			
B	A	-12.864	5	well
B	A			
B	A	-12.946	5	birches
B	A			
B	A	-13.466	5	snug
B	A			
B	A	-15.241	5	blow
B				
B	C	-18.253	5	canei
C				
C		-25.448	5	saxwill

Figure 6a

Cold Hardened Only

Source	DF	SS	MS	F	P
Species	9	102.9545041	11.4393893	15.19	0.0001
Residual	40	30.1325430	0.7533136		
Total	49	133.0870471			

Table 6b

Tukey Grouping		Mean	N	SPECIES
A		-7.4928	5	spin
A				
A		-8.0007	5	canei
A				
B	A	-8.2265	5	saxwp
B	A			
B	A	-8.2865	5	hauy
B	A			
B	C	-10.0508	5	blow
C				
C		-10.7340	5	birches
C				

C -10.8873 5 mela
C
C -10.9284 5 well
C
C -11.1032 5 snug
C
C -11.4022 5 gard
C

Figure 6b

Cold Hardened and Drought Stressed

Source	DF	SS	MS	F	P
Species	11	88.44905217	8.04082292	2.54	0.0128
Residual	48	151.7464878	3.16138516		
Total	59	240.1955400			

Table 6c

Tukey Grouping	Mean	N	SPECIES
A	13.220	5	hauy
A			
A	12.764	5	snug
A			
B A	12.221	5	tour
B A			
B A	11.770	5	birches
B A			
B A	11.708	5	canei
B A			
B A	11.029	5	blow
B A			
B A	11.2916	5	spin
B A			
B A	10.948	5	gard
B A			
B A	10.406	5	well
B A			
B A	10.235	5	mela
B A			
B A	10.085	5	saxwp
B			
B	8.629	5	saxwill

Figure 6c

Out of Season Frost

Source	DF	SS	MS	F	P
Species	11	179.5316080	16.3210553	18.39	0.0001
Residual	48	42.5976987	0.8874521		
Total	59	222.1293067			

Table 6d

Tukey Grouping	Mean	N	SPECIES
A	-5.5106	5	saxwill
B	-7.8690	5	saxwp
B			
C B	-8.1544	5	tour
C B			
C B D	-8.9724	5	hauy
C B D			
C E B D	-9.5494	5	blow
C E B D			
C E B D	-9.7674	5	canei
C E D			
C E D	-9.9579	5	spin
C E D			
C E F D	-10.1919	5	well
E F D			
E F D	-10.5824	5	mela
E F			
E F	-11.3294	5	gard
E F			
E F	-11.4555	5	snug

F
F -12.0077 5 birches

Figure 6d

Cold Hardened at the Springs - 4 weeks (Altitude - 600 m asl)

Source	DF	SS	MS	F	P
Species	11	35.12788014	3.19344365	3.97	0.0004
Residual	48	38.61559584	0.80449158		
Total	59	73.74347598			

Table 6e

Tukey Grouping		Mean	N	SPECIES
A		-9.1900	5	spin
A				
B	A	-10.7151	5	blow
B	A			
B	A	-10.9044	5	well
B				
B		-11.3004	5	hauy
B				
B		-11.5754	5	birches
B				
B		-11.5797	5	gard
B				
B		-11.6152	5	canei
B				
B		-11.6193	5	saxwill
B				
B		-11.6242	5	mela
B				
B		-11.6290	5	saxwp
B				
B		-12.0480	5	tour
B				
B		-12.2830	5	snug

Figure 6e

Cold Hardened at the Springs - 8 weeks (Altitude - 600 m asl)

Source	DF	SS	MS	F	P
Species	11	34.78438288	3.16221663	4.42	0.0003
Residual	36	25.74347362	0.71509649		
Total	47	60.52785650			

Table 6f

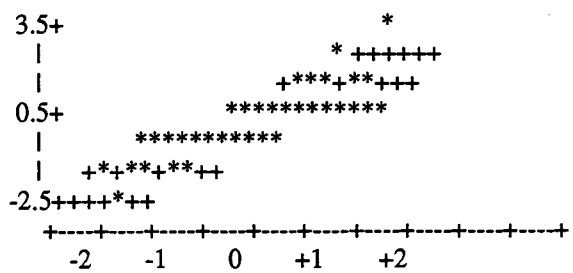
Tukey Grouping		Mean	N	SPECIES
A		-9.1406	4	blow
A				
B	A	-10.630	4	spin
B				
B		-11.2668	4	snug
B				
B		-11.2688	4	mela
B				
B		-11.3104	4	gard

B			
B	-11.5512	4	tour
B			
B	-11.6269	4	saxwp
B			
B	-11.7571	4	birches
B			
B	-11.8655	4	hauy
B			
B	-12.0860	4	well
B			
B	-12.1462	4	canei
B			
B	-12.6366	4	saxwill

Figure 6f

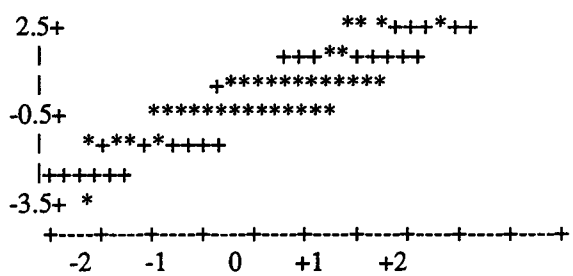
Normal Probability Plots for Probit Transformed Hydroponic Frost Data

Cold Hardened Only



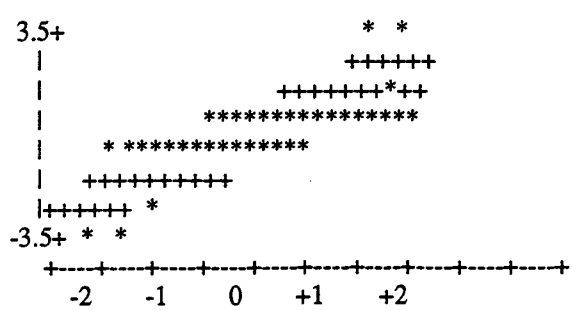
A

Cold Hardened and Drought Hardened



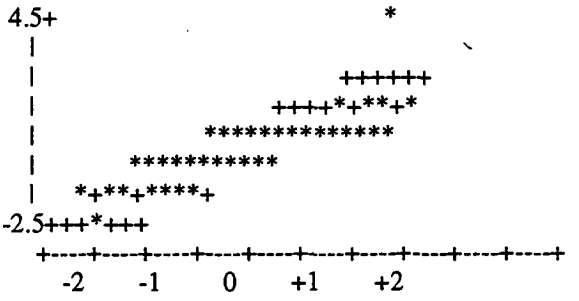
B

Drought Hardened Only



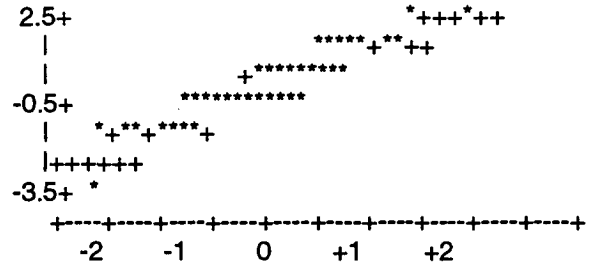
C

Out of Season Frost



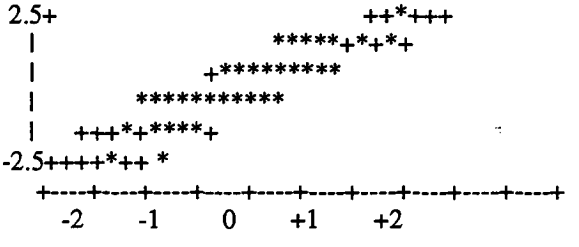
D

Springs 4 weeks



E

Springs 8 weeks



F

APPENDIX 3

Chapter 7 - The Cline ANOVA Tables

Osmotic Potential at Full Turgor

Source	DF	SS	MS	F	P
Site	3	1000.158	333.386	33.58	0.001
Month	13	424.421	32.547	3.29	0.0002
Rep(month)	56	480.045	8.572	0.86	0.7325
Site*Month	39	872.996	22.384	2.25	0.0002
Residual	152	1508.987048	9.927546		
Total	263	4424.566944			

Table 7a

Apoplastic Water Content

Source	DF	SS	MS	F	P
Site	3	3.5865	1.1955	0.89	0.4459
Month	13	31.70003	2.4385	1.82	0.1440
Rep(month)	56	46.0556	0.8224	0.61	0.9809
Site*Month	39	40.7566	1.0450	0.78	0.8145
Residual	152	203.2805402	1.3373720		
Total	263	324.6181745			

Table 7b

Water Potential at Turgor Loss

Source	DF	SS	MS	F	P
Site	3	759.0458	253.0153	5.12	0.0022
Month	13	1883.8661	156.9888	3.18	0.0005
Rep(month)	56	2057.0011	39.5577	0.80	0.8195
Site*Month	39	2530.6359	72.3039	1.46	0.0640
Residual	138	6820.797989	49.426072		
Total	240	14208.62052			

Table 7c

Relative Water Content at Turgor Loss

Source	DF	SS	MS	F	P
Site	3	787.2759	262.4253	10.61	0.001
Month	13	1270.8905	105.9075	4.28	0.0001
Rep(month)	56	1916.1706	36.8494	1.49	0.0352
Site*Month	39	1458.7978	41.6799	1.68	0.0182
Residual	138	3414.302922	24.741326		
Total	240	8518.042988			

Table 7d

Bulk Modulus of Elasticity

Source	DF	SS	MS	F	P
Site	3	110345.7978	36781.9326	4.37	0.0056
Month	13	55747.3922	4288.2609	0.51	0.9158
Rep(month)	56	390159.6685	6967.1369	0.83	0.7877
Site*Month	39	320660.2473	8438.4276	1.00	0.4752
Residual	143	1202913.462	8411.9823		
Total	253	2104853.860			

Table 7e

Water Relations ANOVA Tables site by site

Kingston Beach

Osmotic Potential at Full Turgor

Source	DF	SS	MS	F	P
Month	13	461.629395	35.5099535	2.67	0.0063
Residual	51	679.3589568	13.3207639		
Total	64	1140.988352			

Table 7.1a

Apoplastic Water Content

Source	DF	SS	MS	F	P
Month	13	11.28621483	0.86817037	0.98	0.4828
Residual	51	45.15841622	0.88545914		
Total	64	56.44463105			

Table 7.1b

Water Potential at Turgor Loss

Source	DF	SS	MS	F	P
Month	12	1066.544809	88.878734	1.32	0.2397
Residual	48	3236.26666	67.4222222		
Total	60	4302.81147			

Table 7.1c

%Relative Water Content at Turgor Loss

Source	DF	SS	MS	F	P
Month	12	919.2957284	76.6079774	2.25	0.0235
Residual	48	1632.678566	34.0141368		
Total	60	2551.974295			

Table 7.1d

Bulk Modulus of Elasticity

Source	DF	SS	MS	F	P
Month	13	163729.3292	12594.5638	0.85	0.6114
Residual	50	744263.9914	14885.2798		
Total	63	907993.3206			

Table 7.1e

University

Osmotic Potential at Full Turgor

Source	DF	SS	MS	F	P
Month	13	99.78671536	7.67590118	0.91	0.5471
Residual	53	446.2261910	8.41936209		
Total	66	546.0129063			

Table 7.2a

Apoplastic Water Content

Source	DF	SS	MS	F	P
Month	13	11.72380914	0.90183147	0.87	0.5881
Residual	53	54.96288480	1.03703556		
Total	66	66.68669394			

Table 7.2b

Water Potential at Turgor Loss

Source	DF	SS	MS	F	P
Month	11	251.5849371	22.87135790	0.94	0.5151
Residual	46	1123.341930	24.4204768		
Total	57	1374.926867			

Table 7.2c

%Relative Water Content at Turgor Loss

Source	DF	SS	MS	F	P
Month	11	211.4235517	19.2203229	0.88	0.5631
Residual	46	1001.658000	21.7751739		
Total	57	1213.081551			

Table 7.2d

Bulk Modulus of Elastcity

Source	DF	SS	MS	F	P
Month	12	206591.0968	17215.9247	1.04	0.4257
Residual	50	824606.2788	16492.1256		
Total	62	1031197.375			

Table 7.2e

Mount Nelson

Osmotic Potential at Full Turgor

Source	DF	SS	MS	F	P
Month	13	608.6088718	46.8160671	6.83	0.0001
Residual	53	363.1413926	6.8517244		
Total	66	971.7502645			

Table 7.3a

Apoplastic Water Content

Source	DF	SS	MS	F	P
Month	13	48.54004788	3.73384984	1.34	0.2219
Residual	53	147.9832210	2.79213625		
Total	66	196.5232689			

Table 7.3b

Water Potential at Turgor Loss

Source	DF	SS	MS	F	P
Month	12	1309.884490	109.157041	5.69	0.0001
Residual	49	940.453076	19.192920		
Total	61	2250.337565			

Table 7.3c

%Relative Water Content at Turgor Loss

Source	DF	SS	MS	F	P
Month	12	943.1522742	78.5960228	4.41	0.0001
Residual	49	873.8995000	17.8346837		
Total	61	1817.051774			

Table 7.3d

Bulk Modulus of Elastcity

Source	DF	SS	MS	F	P
Month	13	12154.99551	934.99965	3	0.0025
Residual	51	15873.34383	311.24204		
Total	64	28028.33934			

Table 7.3e

Mount Wellington

Osmotic Potential at Full Turgor

Source	DF	SS	MS	F	P
Month	13	196.5770756	15.1213135	1.54	0.1349
Residual	51	500.3059079	9.8099198		
Total	64	696.8829835			

Table 7.4a

Apoplastic Water Content

Source	DF	SS	MS	F	P
Month	13	0.65971970	0.05074767	2.10	0.302
Residual	51	1.23165110	0.02415002		
Total	64	1.89137080			

Table 7.4b

Water Potential at Turgor Loss

Source	DF	SS	MS	F	P

Month	12	1778.912500	148.242708	1.95	0.0522
Residual	47	3577.737500	76.122074		
Total	59	5356.650000			

Table 7.4c

%Relative Water Content at Turor Loss

Source	DF	SS	MS	F	P
Month	12	467.5883333	38.9656944	1.01	0.4594
Residual	47	1822.237500	38.7710106		
Total	59	2289.825833			

Table 7.4d

Bulk Modulusof Elasticity

Source	DF	SS	MS	F	P
Month	13	5464.083195	420.314092	2.42	0.0132
Residual	48	8329.516775	173.531599		
Total	61	13793.59997			

Table 7.4e

Tukey's Test

Kingston Beach

Osmotic Potential at Full Turgor

Tukey Grouping		Mean	N	MONTH
A		-9.580	4	jul
A				
B	A	-14.030	5	feb
B	A			
B	A	-15.636	5	dec
B	A			
B	A	-15.702	5	june
B	A			
B	A	-16.326	5	ap
B	A			
B	A	-17.210	5	may
B	A			
B	A	-17.293	5	augb
B	A			
B	A	-17.328	5	mar
B				
B		-18.712	4	aug
B				
B		-19.269	5	jul94
B				
B		-19.633	5	nov
B				
B		-19.920	3	jan
B				
B		-20.140	5	sep
B				
B		-20.130	4	oct

Figure 7.1a

Apoplastic water Content

Tukey Grouping	Mean	N	MONTH
A	0.6587	4	jul
A			
A	0.4757	5	nov
A			
A	0.4579	4	aug
A			
A	0.4097	5	augb
A			
A	0.3857	5	june
A			
A	0.3699	5	julb
A			
A	0.3607	5	may
A			
A	0.3551	5	ap
A			
A	0.3507	5	feb
A			

A	0.3440	5	mar
A			
A	0.334	4	oct
A			
A	0.3282	3	jan
A			
A	0.2908	5	sep
A			
A	0.2283	5	dec

Figure 7.1b

Water Potential at Turgor Loss

Tukey Grouping	Mean	N	MONTH
A -	-15.750	4	jul
A			
A	-19.500	5	sep
A			
A	-22.100	5	june
A			
A	-22.700	5	dec
A			
A	-23.300	5	mar
A			
A	-26.000	5	may
A			
A	-26.100	5	ap
A			
A	-27.000	5	augb
A			
A	-27.400	5	julb
A			
A	-27.600	5	feb
A			
A	-30.250	4	oct
A			
A	-30.600	5	nov
A			
A	-31.667	3	jan

Figure 7.1c

Relative Water Content at Turgor Loss

Tukey Grouping	Mean	N	MONTH
A	86.075	4	jul
A			
B A	83.400	5	augb
B A			
B A	82.580	5	june
B A			
B A	81.920	5	julb
B A			
B A	80.300	5	sep
B A			
B A	78.950	4	oct
B A			
B A	78.900	5	may
B A			

B	A	78.800	5	mar
B	A			
B	A	78.440	5	ap
B	A			
B	A	78.320	5	dec
B	A			
B	A	74.587	3	jan
B	A			
B	A	73.500	5	nov
B				
B		71.340	5	feb

Figure 7.1d

Bulk Elastic Modulus

Tukey Grouping	Mean	N	MONTH
A	73.27	3	jan
A			
A	70.61	4	aug
A			
A	60.01	4	oct
A			
A	58.84	5	sep
A			
A	58.66	5	jul94
A			
A	52.41	4	ap
A			
A	47.87	5	dec
A			
A	47.17	5	may
A			
A	45.21	5	nov
A			
A	42.24	5	mar
A			
A	41.767	5	feb
A			
A	40.35	5	aug94
A			
A	37.93	5	june
A			
A	17.50	4	jul

Figure 7.1e

University

Osmotic Potential at Full Turgor

Tukey Grouping	Mean	N	MONTH
A	-16.624	5	jul94
A			
A	-18.142	5	mar
A			
A	-18.378	5	dec
A.			
A	-18.440	4	jan
A			

A	-18.600	5	june
A			
A	-19.060	5	feb
A			
A	-19.098	4	jul
A			
A	-19.289	5	sep
A			
A	-19.440	5	ap
A			
A	-19.480	5	aug94
A			
A	-19.492	5	may
A			
A	-20.127	4	oct
A			
A	-20.954	5	aug
A			
A	-21.794	5	nov

Figure 7.2a

Apoplastic Water Content

Tukey Grouping	Mean	N	MONTH
A	0.5040	5	sep
A			
A	0.4220	5	feb
A			
A	0.4038	5	aug94
A			
A	0.4022	5	may
A			
A	0.3953	5	jul94
A			
A	0.3866	5	june
A			
A	0.3823	4	jul
A			
A	0.3811	5	dec
A			
A	0.3583	4	oct
A			
A	0.3476	5	ap
A			
A	0.3450	4	jan
A			
A	0.3362	5	mar
A			
A	0.3070	5	nov
A			
A	0.2323	5	aug

Figure 7.2b

Water Potential at Turgor loss

Tukey Grouping	Mean	N	MONTH
A			
A	-26.200	5	feb

A			
A	-26.912	5	dec
A			
A	-27.000	5	sep
A			
A	-27.900	5	mar
A.			
A	-28.000	5	june
A			
A	-28.900	5	may
A			
A	-29.000	4	jan
A			
A	-29.300	5	jul94
A			
A	-30.300	5	nov
A			
A	-30.725	4	oct
A			
A	-32.200	5	ap
A			
A	-33.200	5	aug94

Figure 7.2c

University

Relative Water Content at Turgor Loss

Tukey Grouping	Mean	N	MONTH
A	81.960	5	feb
A			
A	81.500	5	sep
A			
A	81.020	5	dec
A			
A	80.960	5	may
A			
A	80.050	4	oct
A			
A	79.380	5	june
A			
A	78.380	5	jul94
A			
A	77.700	4	jan
A			
A	77.420	5	nov
A			
A	77.340	5	aug94
A			
A	76.940	5	mar
A			
A	76.320	5	ap

Figure 7.2d

Bulk Modulus of Elasticity

Tukey Grouping	Mean	N	MONTH
A			

A	93.70	5	nov
A			
A	75.62	5	aug
A			
A	67.27	5	may
A			
A	58.78	5	sep
A			
A	54.05	4	jan
A			
A	53.98	5	ap
A			
A	50.13	5	mar
A.			
A	50.10	4	oct
A			
A	49.94	5	feb
A			
A	49.45	5	june
A			
A	46.36	5	aug94
A			
A	45.69	5	jul94
A			
A	43.27	5	dec
A			
A	32.46	5	jul

Figure 7.2e

Mount Nelson

Osmotic Potential at Full Turgor

Tukey Grouping	Mean	N	MONTH
A	-9.128	4	jul
A			
B	-16.625	5	may
B			
B	-18.384	5	jul94
B			
B	-19.114	5	dec
B			
B	-19.170	5	june
B			
B	-19.460	5	ap
B			
B	-19.485	5	nov
B			
B	-20.122	5	aug94
B			
B	-20.790	4	feb
B.			
B	-20.832	5	oct
B			
B	-21.134	5	jan
B			
B	-22.195	4	aug
B			
B	-22.207	5	sep
B			

B	-22.243	5	mar

Figure 7.3a

Apoplastic Water Content

Tukey Grouping	Mean	N	MONTH
A	0.784	4	jul
A			
A	0.430	5	may
A			
A	0.398	5	jul94
A			
A	0.371	5	june
A			
A	0.368	4	aug
A			
A	0.354	5	ap
A			
A	0.330	5	mar
A			
A	0.324	5	nov
A			
A	0.305	5	jan
A			
A	0.276	5	dec
A			
A	0.275	5	oct
A			
A	0.260	5	sep
A			
A	0.258	5	aug94
A			
A	0.220	4	feb

Figure 7.3b

Water Potential at Turgor Loss

Tukey Grouping		Mean	N	MONTH
A		-15.313	4	jul
A				
B		-27.200	5	jul94
B				
C	B	-27.400	5	nov
C	B			
C	B	-28.700	5	dec
C	B			
C	B	-29.000	5	june
C	B			
C	B	-29.200	5	may
C	B			
C	B	-29.625	4	feb
C	B			
C	B	-30.300	5	oct
C	B			
C	B	-30.700	5	sep
C	B			
C	B	-31.600	5	aug94
C	B			

C	B	-31.625	4	ap
C	B			
C	B	-33.900	5	jan
C				
C		-37.300	5	mar

Figure 7.3c

%Relative Water Content at Turgor Loss

Tukey Grouping		Mean	N	MONTH
A		89.650	4	jul
A				
B	A	80.580	5	jul94
B				
B		79.620	5	may
B				
B		79.580	5	sep
B				
B		79.000	5	june
B				
B		78.897	5	nov
B				
B		78.300	5	dec
B				
B		75.980	5	oct
B				
B		75.040	5	aug94
B				
B		74.575	4	feb
B				
B		74.220	5	jan
B				
B		74.150	4	ap
B				
B		73.720	5	mar

Figure 7.3d

Bulk Modulus of Elasticity

Tukey Grouping	Mean	N	MONTH
A	67.41	5	sep
A			
A	64.97	4	aug
A			
A	64.77	3	feb
A			
A	59.71	5	jan
A			
A	58.27	5	dec
A			
A	57.76	5	nov
A			
A	54.81	5	aug94
A			
A	54.17	5	oct
A			
A	52.81	5	june
A			

B	A	49.56	5	jul94
B	A			
B	A	44.62	5	may
B	A			
B	A	43.27	4	ap
B	A			
B	A	35.44	5	mar
B				
B		9.79	4	jul

Figure 7.3e

Mount Wellington

Osmotic Potential at Turgor Loss

Tukey Grouping	Mean	N	MONTH
A	-10.870	5	jan
A			
A	-12.426	5	june
A			
A	-13.184	5	mar
A			
A	-13.685	4	nov
A			
A	-13.807	5	ap
A			
A	-13.937	4	aug
A			
A	-14.026	5	feb
A			
A	-14.054	4	aug94
A			
A	-14.171	4	sep
A			
A	-15.548	4	may
A			
A	-15.578	5	jul94
A			
A	-15.700	5	dec
A			
A	-16.674	5	oct
A			
A	-17.712	5	jul

Figure 7.4a

Apoplastic Water Content

Tukey Grouping	Mean	N	MONTH
A	0.6475	4	aug
A			
A	0.5139	4	aug94
A			
A	0.5139	5	jul94
A			
A	0.4778	4	sep
A			
A	0.4697	5	ap
A			

A		0.4681	5	jan
A				
A		0.4630	5	june
A				
A		0.4470	4	may
A				
A		0.4370	4	nov
A				
A		0.4353	5	oct
A				
A		0.4348	5	mar
A				
A		0.4229	5	jul
A				
A		0.2421	5	feb
A				
A		0.2283	5	dec

Figure 7.4b

Water Potential at Turgor Loss

Tukey Grouping		Mean	N	MONTH
A		-11.625	4	sep
A				
B	A	-20.800	5	feb
B	A			
B	A	-22.100	5	june
B	A			
B	A	-22.700	5	dec
B	A			
B	A	-23.400	5	mar
B	A			
B	A	-23.875	4	nov
B	A			
B	A	-25.375	4	ap
B	A			
B	A	-28.000	5	jan
B	A			
B	A	-28.375	4	may
B	A			
B	A	-30.200	5	jul94
B	A			
B	A	-30.625	4	aug94
B	A			
B	A	-30.900	5	jul
B				
B		-33.800	5	oct

Figure 7.4c

%Relative Water Content at Turgor Loss

Tukey Grouping	Mean	N	MONTH
A	78.320	5	dec
A			
A	77.880	5	feb
A			
A	77.420	5	jul94
A			

A		76.700	5	june
A				
A		76.425	4	ap
A				
A		76.400	4	sep
A				
A		75.125	4	nov
A				
A		75.025	4	may
A				
A		74.680	5	mar
A				
A		73.980	5	jul
A				
A		73.925	4	aug94
A				
A		72.220	5	oct
A				
A		67.760	5	jan

Figure 7.4d

Bulk Modulus of Elasticity

Tukey Grouping		Mean	N	MONTH
A		29.380	4	aug
A				
B	A	24.997	4	may
B	A			
B	A	22.168	4	ap
B	A			
B	A	20.646	5	mar
B	A			
B	A	20.277	5	jul94
B	A			
B	A	18.935	4	aug94
B	A			
B	A	18.803	4	oct
B	A			
B	A	17.477	4	nov
B	A			
B	A	13.898	4	feb
B	A			
B	A	12.547	5	june
B	A			
B	A	11.496	4	sep
B	A			
B	A	4.823	5	jan
B	A			
B	A	3.44	5	dec
B				
B		1.15	5	jul

Figure 7.4e

Cline Frost Results

ANOVA tables

Source	DF	SS	MS	F	P
Site	5	83.36459192	16.67291838	12.74	0.0001

Month	12	417.5633643	34.79694703	26.59	0.0001
Rep(Month)	52	136.4101883	2.62327285	2	0.0002
Site*Month	60	148.8076565	2.48012761	1.90	0.0004
Residual	245	320.6082178	1.30860497		
Total	374	1106.754019			

Table 7

Kingston Beach

Source	DF	SS	MS	F	P
Month	12	155.9063947		12.99219956	0.0001
Residual	51	92.08641623	1.80561600		
Total	63	247.9928110			

Table 7a

Mount Nelson

Source	DF	SS	MS	F	P
Month	12	96.43139097	8.03594925	3.97	0.0003
Residual	51	103.3103478	2.02569309		
Total	63	199.7417388			

Table 7b

Ridgeway Reserve

Source	DF	SS	MS	F	P
Month	12	69.24571609	5.77047634	4.86	0.0001
Residual	50	59.36649592	1.18732992		
Total	62	1128.612212			

Table 7c

University

Source	DF	SS	MS	F	P
Month	12	70.15209167	5.84600764	3.75	0.0005
Residual	49	76.32390836	1.55763078		
Total	61	146.4760000			

Table 7d

Waterworks

Source	DF	SS	MS	F	P
Month	12	96.3851694	8.0320974	13.74	0.0001
Residual	49	28.63796495	0.58444826		
Total	61	125.0231343			

Table 7e

Mount Wellington

Source	DF	SS	MS	F	P
Month	12	83.3776883	6.94814069	3.54	0.0009
Residual	47	92.1658425	1.96097537		
Total	59	175.5435308			

Table 7f

Tukey's Test

Kingston Beach

Tukey Grouping	Mean	N	MONTH
A	-6.0444	5	jul
A			
B A	-7.8744	5	aug
B A			
B A C	-8.3959	5	oct
B C			
B C	-9.9163	5	nov
B C			
B C	-10.1892	5	Ap
B C			
B C	-10.3340	5	jul94
B C			
B C	-10.5275	5	sep
C			
C	-10.8458	5	May
C			
C	-11.1110	5	jun
C			
C	-11.1714	4	dec
C			
C	-11.1891	5	jan
C			
C	-11.2032	5	aug94
C			
C	-11.2874	5	mar

Figure 7a

Mount Nelson

Tukey Grouping	Mean	N	MONTH
A	-7.8401	5	oct
A			
B A	-9.0951	5	nov
B A			
B A	-9.1797	5	aug
B A			
B A	-9.9921	5	sep
B A			
B A	-10.4507	5	aug94
B A			
B A	-10.5960	5	jul94
B A			
B A	-10.8087	5	jul
B			
B	-11.0616	5	dec
B			
B	-11.4616	5	Ap
B			
B	-11.6998	4	jan
B			

B	-11.7529	5	may
B			
B	-11.8891	5	jun
B			
B	-12.0282	5	mar

Figure 7b

Ridgeway Reserve

Tukey Grouping	Mean	N	MONTH
A	-9.1548	5	aug
A			
B A	-9.3094	5	nov
B A			
B A	-9.3823	4	oct
B A			
B A C	-9.8336	5	jul
B A C			
B D A C	-10.0369	5	dec
B D A C			
B D A C	-10.1593	4	sep
B D A C			
B D A C	-10.9938	5	jul94
B D A C			
B D A C	-11.2382	5	aug94
B D A C			
B D A C	-11.3592	5	jun
B D A C			
B D A C	-11.6239	5	jan
B D C			
B D C	-11.6339	5	Ap
D C			
D C	-11.9719	5	May
D			
D	-12.4018	5	mar

Figure 7c

University

Tukey Grouping	Mean	N	MONTH
A	-8.5171	4	aug
A			
B A	-9.2703	4	nov
B A			
B A	-9.5189	5	oct
B A			
B A	-9.7137	5	jul
B A			
B A	-10.3175	5	mar
B A			
B A	-10.3273	5	sep
B A			
B A	-10.5871	4	jan
B A			
B A	-11.0563	5	jul94
B A			
B A	-11.2691	5	May
B			

B	-11.5715	5	aug94
B			
B	-11.7902	5	Ap
B			
B	-11.9912	5	jun
B			
B	-12.0654	5	dec

Figure 7d

Waterworks

Tukey Grouping	Mean	N	MONTH
A	-7.6812	5	aug
A			
A	-7.9850	4	jul
A			
B A	-8.6565	5	nov
B A			
B A C	-9.3380	4	jan
B C			
B D C	-9.7498	5	oct
B D C			
B E D C	-10.1986	5	mar
E D C			
E D C	-10.3876	5	sep
E D C			
E D C	-10.5762	5	jul94
E D C			
E D C	-10.5933	4	dec
E D C			
E D C	-10.8403	5	May
E D			
E D	-11.2804	5	aug94
E D			
E D	-11.4459	5	Ap
E			
E	-11.8079	5	jun

Figure 7e

Mount Wellington

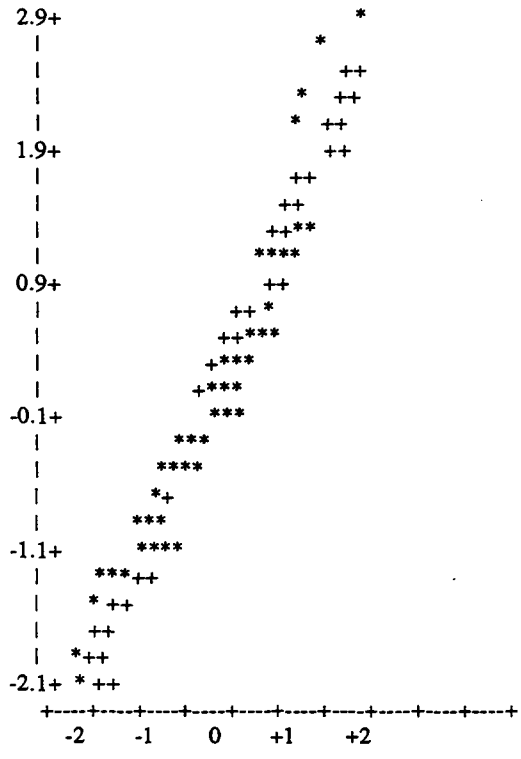
Tukey Grouping	Mean	N	MONTH
A	-9.0286	4	jul
A			
B A	-9.7871	5	aug
B A			
B A	-10.3556	5	oct
B A			
B A	-10.7414	4	nov
B A			
B A	-10.7917	4	jan
B A			
B A	-10.9922	4	sep
B A			
B A	-11.1817	5	dec
B A			
B A	-12.0391	5	May
B			

B	-12.3717	5	mar
B			
B	-12.3894	5	jul94
B			
B	-12.6265	5	aug94
B			
B	-12.7126	4	Ap
B			
B	-12.9671	5	jun

Figure 7f

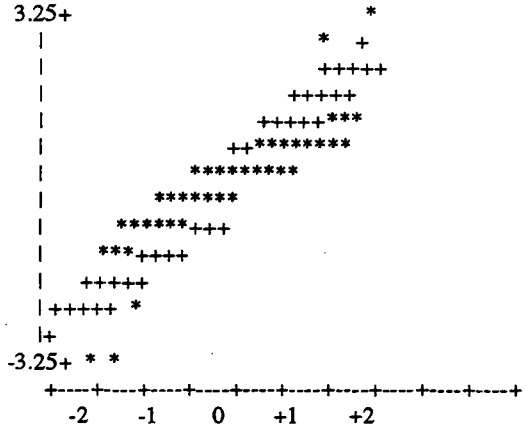
Cline Frost Normal Probability Plot for probit transformed data

Kingston Beach



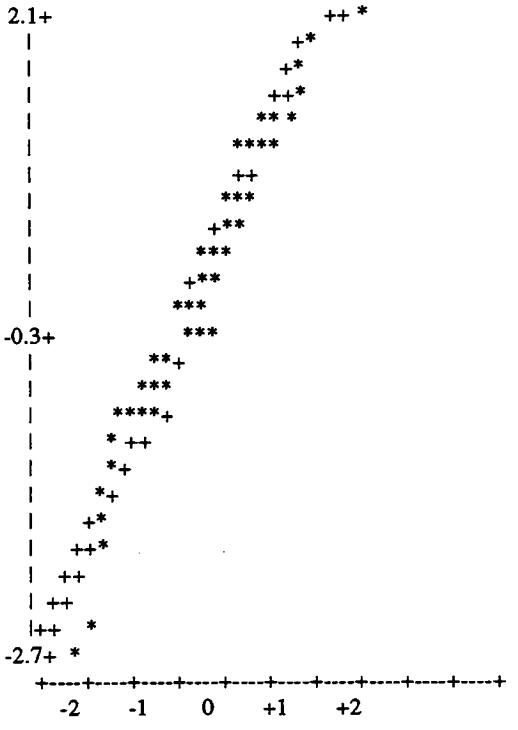
A

Mount Nelson



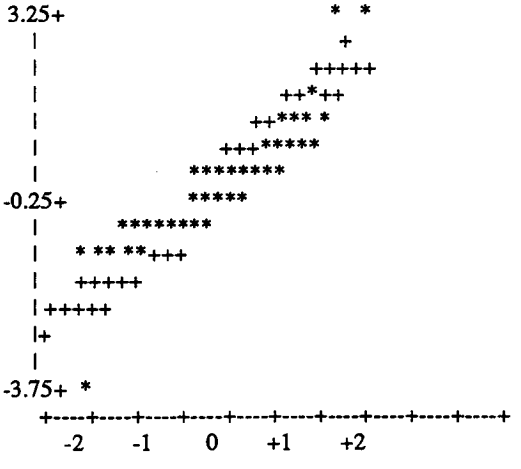
B

Ridgeway



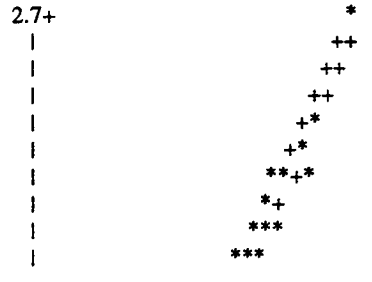
C

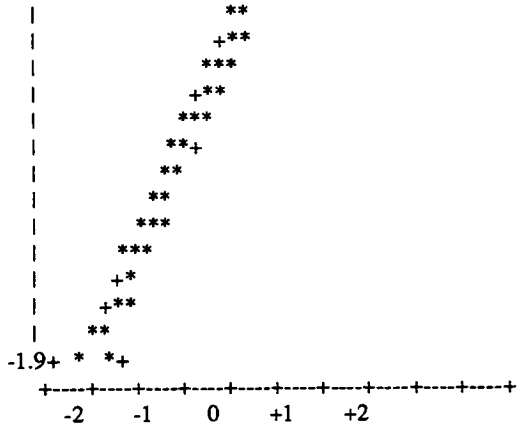
University



D

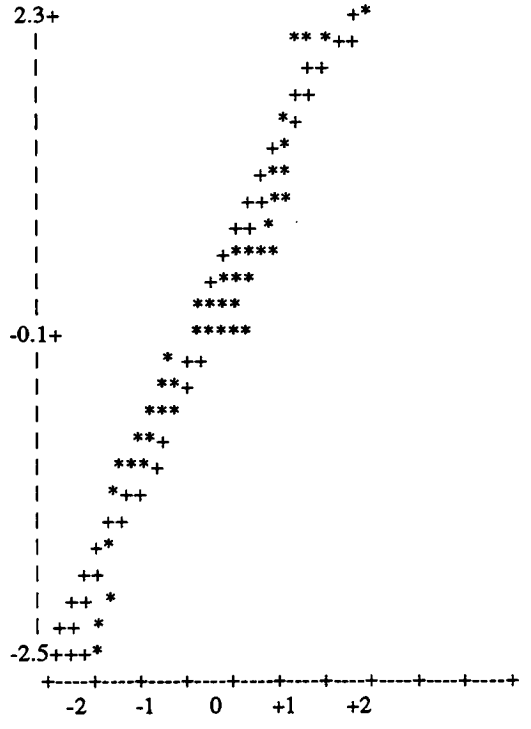
Waterworks





E

Mount Wellington



F

APPENDIX 4

Chapter 9 - Intrapopulation Variation within Five Populations of *Banksia marginata*

Analysis of Variance Tables

Desiccation

Source	DF	SS	MS	F	P
Site	4	982.9677179	245.7419295	2.08	0.1122
Tree(Site)	25	4255.567241	170.2226897	1.44	0.1800
Residual	26	3070.123370	118.0816681		
Total	55	8308.658330			

Table 9a

Frost

Source	DF	SS	MS	F	P
Site	4	134.9208450	33.73021125	3.60	0.0164
Tree(Site)	25	525.4879730	21.01951892	2.74	0.0179
Residual	30	281.1964505	9.37321502		
Total	59	941.6052685			

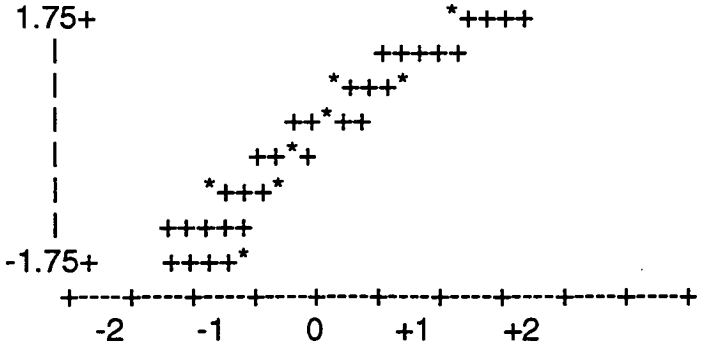
Table 9b

Chapter 9

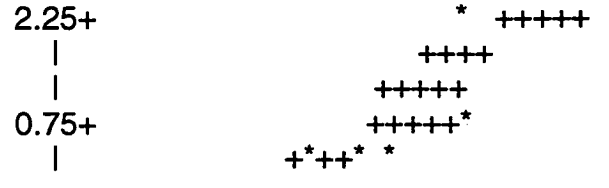
Desiccation Experiment

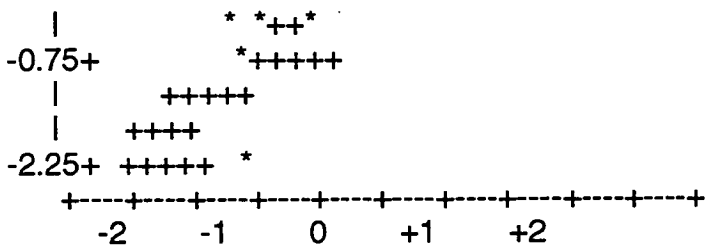
Normal Probability plots for probit transformed data

Primrose 1

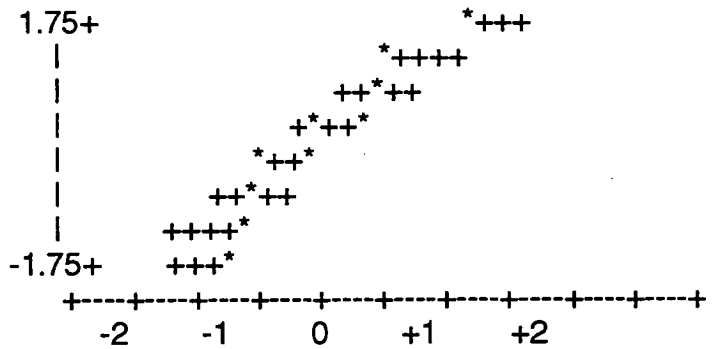


Primrose 2

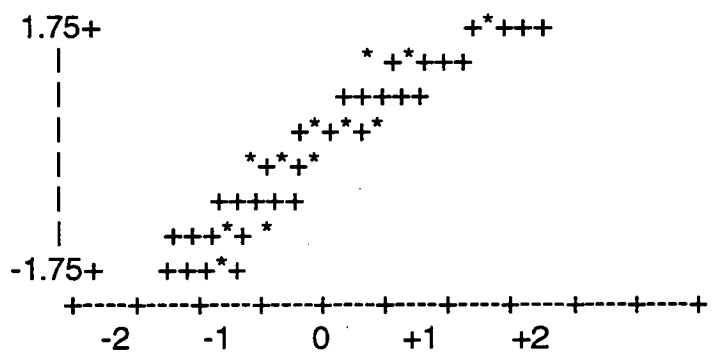




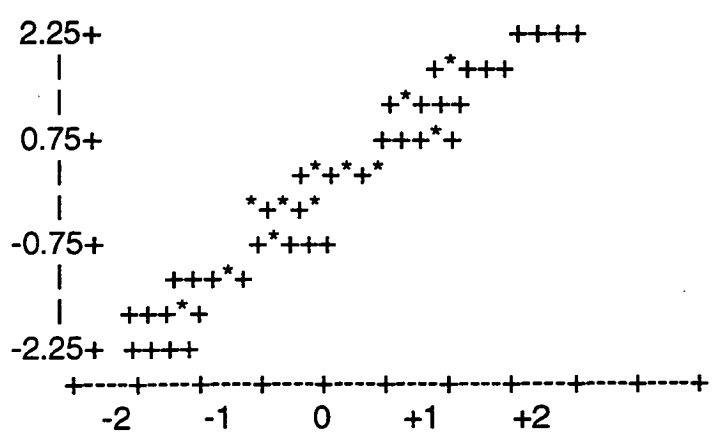
Ridgeway 1



Ridgeway 2



Southarm



Please note: I do not have the normal probability plot for the frost experiment. I left the University of Tasmania and I now live in Brisbane and although it is possible that the file is still on the computer system, it would be very difficult for me to acces. It

would appear that I did not save the output of the probit transformation at the of the analysis. I hope that you will trust that I would have only carried out an ANOVA on normally distributed data.

Appendix 5	Data for isozyme study of *Banksia marginata*				
	(Mt.Wellington and University populations)				
Plant	**Site**	**PER1**	**SKDH1**	**6PGD1**	**6PGD2**
1	UNI	AB	AA	AB	BB
2	UNI	AB	BB	.	AB
3	UNI	AA	AB	AA	AB
5	UNI	AA	AB	AA	AB
6	UNI	AA	AA	AA	BB
7	UNI	AA	AA	BB	BB
8	UNI	AA	AA	BB	AB
9	UNI	AA	AA	AA	AB
10	UNI	AA	AB	AB	AA
11	UNI	BB	AB	BB	.
12	UNI	AB	AB	AA	AB
13	UNI	AA	BB	AA	AB
14	UNI	AA	AA	AA	BB
15	UNI	AA	AB	AA	AB
16	UNI	AA	AA	BB	AA
17	UNI	AB	AB	.	.
19	UNI	AA	AA	AA	AB
20	UNI	AA	AB	AA	AB
21	UNI	AA	AA	AA	AB
22	UNI	AA	AA	AA	AB
23	UNI	AB	AA	AA	.
24	UNI	AA	AB	BB	AA
25	UNI	AB	AB	.	AB
26	UNI	AB	AB	BB	AB
27	UNI	AA	AB	AA	AB
28	UNI	BB	AB	AA	BB
29	UNI	AB	BB	AA	AB
30	UNI	AA	AA	AB	AB
31	UNI	AB	AB	AA	AB
32	UNI	AB	AA	.	AB
34	UNI	AA	AB	AA	AB
35	UNI	AA	AB	AA	AB
36	UNI	AB	AA	AA	AB
37	UNI	AB	BB	AA	AB
38	UNI	AA	AA	BB	AB
39	UNI	AA	AA	AA	AB
40	UNI	AB	AA	AA	AB
41	UNI	AA	AA	AA	BB
42	UNI	AA	AA	AB	AB
43	UNI	AA	BB	AA	BB
44	UNI	AB	AB	AA	AB
45	UNI	AA	AB	.	AB
46	UNI	AB	AB	BB	BB
47	UNI	AB	AB	.	AB
48	UNI	AA	AB	.	BB

49	UNI	AA	AB	AB	AB
51	UNI	AB	AB	AB	AB
1	WEL	AB	AB	AA	BB
2	WEL	AA	BB	AA	BB
3	WEL	AA	BB	BB	BB
4	WEL	BB	AB	BB	AB
5	WEL	AB	AA	AA	BB
6	WEL	AA	BB	BB	BB
7	WEL	AA	AB	AA	AB
8	WEL	AA	AB	AA	BB
9	WEL	BB	BB	AA	BB
10	WEL	AA	AB	AB	BB
12	WEL	AA	BB	AB	BB
13	WEL	AB	AB	AB	BB
14	WEL	AA	BB	BB	AB
15	WEL	AB	BB	BB	BB
16	WEL	AA	AB	AA	BB
17	WEL	AA	BB	AA	BB
18	WEL	AA	BB	AA	.
19	WEL	AA	AB	AA	AB
20	WEL	AA	BB	AB	BB
21	WEL	AA	AB	AB	AB
22	WEL	BB	BB	AB	BB
25	WEL	AA	BB	AB	AB
26	WEL	AA	AB	BB	BB
27	WEL	AB	AB	AB	BB
28	WEL	AA	AB	AA	BB
29	WEL	AA	BB	BB	AA
30	WEL	AB	AA	AB	AB
31	WEL	AB	BB	AB	BB
32	WEL	AA	AB	BB	BB
33	WEL	AA	BB	AB	BB
34	WEL	AB	BB	AA	BB
35	WEL	AA	BB	AA	BB
36	WEL	BB	AB	AB	AA
37	WEL	AA	AB	AB	AB
38	WEL	AB	BB	AA	BB
39	WEL	AA	BB	AB	AB
40	WEL	AA	AB	AB	AB
41	WEL	AA	AB	AA	AB
42	WEL	AA	BB	AB	BB
43	WEL	AB	AB	AB	BB
44	WEL	AB	AB	BB	.
45	WEL	AA	BB	AB	BB
46	WEL	AA	BB	AB	AB
47	WEL	AA	AB	AA	BB
48	WEL	AB	AB	BB	BB
49	WEL	AA	BB	AB	BB
50	WEL	AB	AB	AA	BB
51	WEL	AA	AB	AA	AB

52	WEL	BB	AB	AB	AB
53	WEL	AA	BB	BB	BB
54	WEL	AA	BB	AA	AA